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OPINION REVIEW

Approach to medical therapy in perianal Crohn's disease

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Abstract

Perianal Crohn's disease remains a challenging condition to treat and can have a substantial negative impact on quality of life. It often requires combined surgical and medical interventions. Anti-tumor necrosis factor (anti-TNF) therapy, including infliximab and adalimumab, remain preferred medical therapies for perianal Crohn's disease. Infliximab has been shown to be efficacious in improving fistula closure rates in randomized controlled trials. Clinicians can be faced with a number of questions relating to the optimal use of anti-TNF therapy in perianal Crohn's disease. Specific issues include evaluation for the presence of perianal sepsis, the treatment target of therapy, the ideal time to commence treatment, whether additional medical therapy should be used in conjunction with anti-TNF therapy, and the duration of treatment. This article will discuss key studies which can assist clinicians in addressing these matters when they are considering or have already commenced anti-TNF therapy for the treatment of perianal Crohn's disease. It will also discuss current evidence regarding the use of vedolizumab and ustekinumab in patients who are failing to achieve a response to anti-TNF therapy for perianal Crohn's disease. Lastly, new therapies such as local injection of mesenchymal stem cell therapy will be discussed.

Key Words: Fistula; Biologics; Inflammatory bowel disease; Surgery; Stem cells; Infliximab; Ustekinumab

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Core Tip: Early commencement of anti-tumor necrosis factor (anti-TNF) therapy in perianal Crohn's disease is preferred over delaying treatment, although perianal sepsis should be treated first. Symptomatic remission remains the treatment goal, with

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radiographic healing an evolving target. Concomitant antibiotic therapy while initiating anti-TNF therapy is efficacious. Therapeutic drug monitoring and dose adjustment of anti-TNF therapy, targeting a higher trough level than what is routinely used for luminal disease, may improve treatment response. Ustekinumab may be efficacious in anti-TNF refractory individuals, although more studies are needed. Mesenchymal stem cell injection can be used in individuals who are refractory to anti-TNF therapy.

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INTRODUCTION

Perianal Crohn's disease is a debilitating complication of Crohn's disease, occurring in up to 30% of individuals with luminal disease[1-3]. It remains a difficult condition to treat, with relapses occurring frequently, and it is associated with a higher rate of hospitalization and surgery compared to individuals with Crohn's disease without perianal disease[4-6]. Perianal Crohn's disease is also associated with a significant burden on quality of life with far reaching consequences on the well-being of an individual, including affecting social relationships and work-related opportunities[7]. Treatment often requires multimodal therapy with surgical treatment and systemic medical therapy used in combination, and this has been shown to be more efficacious than either strategy alone[8,9]. Significant advancements have been made in recent times with surgical and local therapy for perianal disease, with local injections of mesenchymal stem cell therapy showing remarkable promise in randomized studies [10,11]. Despite the advancements in surgical techniques and local therapy in perianal disease and the plethora of medical therapies that are being explored for the treatment of luminal Crohn's disease, systemic medical therapy in perianal Crohn's disease has been dominated by anti-tumor necrosis factor (TNF) therapy, which was shown to be efficacious over two decades ago[12]. Infliximab is the only biologic treatment that has been proven efficacious in a dedicated randomized placebo-controlled trial in individuals with symptomatic perianal Crohn's disease.

While the efficacy of anti-TNF therapy has been established, there is a wide variation in the management of perianal Crohn's disease amongst treating clinicians [13]. This may partially relate to the fact that there are a number of situations that the treating clinician may encounter that are not addressed by randomized studies and where evidence is either insufficient or evolving. These include the timing and duration of anti-TNF therapy, the need for concomitant or increased dose of therapy and what alternative biologics can be used in an individual who is failing anti-TNF therapy. While conclusive data may not be available in many of these situations, there are studies that have been conducted that may provide further insight into decision making in these situations. The aim of this paper, therefore, is to address these potential questions that may assist treating clinicians who are considering medical therapy or have commenced anti-TNF therapy for the treatment of perianal Crohn's disease.

WHAT MODALITIES SHOULD I USE TO EVALUATE PERIANAL CROHN'S DISEASE PRIOR TO COMMENCING ANTI-TNF THERAPY?

An important component of medical therapy when treating perianal Crohn's disease is to control underlying infection prior to commencing immunosuppressive therapy. There are several modalities that can be utilized to evaluate for the presence of a perianal abscess or deep infection, including examination under anesthesia (EUA), magnetic resonance imaging (MRI) pelvis and transrectal ultrasound (TRUS). All modalities have been shown to correctly classify Crohn's perianal fistulae in over 85% of patients and when two modalities are used, the accuracy approaches 100%[14].

Transperineal ultrasound, which is a non-invasive and relatively cheaper modality of imaging, has also been shown to have similar accuracy to TRUS and MRI pelvis in diagnosing perianal fistulae[15]. If available, pelvic MRI provides the most comprehensive evaluation of the perianal region (Figure 1). Small field of view images accurately depict even the most complex fistulous tracts as well as the size, number and location of an abscess cavities including disease extending above the levator ani musculature. Associated features, such as rectal inflammation and lymphadenopathy, can also be seen. Use of one or more of these forms of diagnostic imaging or an EUA should be performed prior to commencing medical therapy with an anti-TNF for the treatment of perianal Crohn's disease.

WHEN IS THE BEST TIME TO COMMENCE ANTI-TNF THERAPY IN PERIANAL CROHN'S DISEASE?

A retrospective claims-based study evaluated the role of early use of immunosuppressive therapies in reducing the risk of developing future perianal disease amongst indi-viduals with a new diagnosis of Crohn's disease. In this study, individuals with a new diagnosis of Crohn's disease without perianal disease exposed to at least 90 days of immunosuppressants or anti-TNF therapy had a 59% reduction in risk, compared to those not using these agents, of developing symptomatic perianal disease over 2 years [16]. While these results require further validation, this study raises the possibility that individuals who are identified as having perianal disease may benefit from earlier medical therapy with immunosuppressive or anti-TNF therapy even if they are minimally symptomatic.

In contrast to these findings, data would suggest that there is often a significant delay in the commencement of biologic therapy in patients with perianal Crohn's disease, with a median of over 6 months between the initial diagnosis of perianal Crohn's disease and the commencement of anti-TNF therapy[17]. The concern for worsening perianal infection may partially explain this delay of immunosuppressive treatment. A retrospective study compared fistula recurrence rates amongst 76 individuals with perianal Crohn's disease who were either started on infliximab late (median, 250 days) or early (median, 12 days) after seton insertion for perianal Crohn's disease[18]. Recurrence rates were low in both groups of this study (8% overall), and no difference was found between early and late commencement of infliximab and no reports of abscesses or perianal sepsis in either group.

A multicenter randomized study assigned patients with newly diagnosed and recurrent perianal Crohn's disease with a seton inserted and fistula drainage to one of three groups: chronic fistula drainage for 12 months, anti-TNF therapy (adalimumab or infliximab) for 12 months with removal of seton at 6 weeks, or initial anti-TNF therapy followed by surgical closure of the fistula within 8 weeks to 12 weeks[19]. The study was initially designed to show superiority of seton insertion in reducing future surgical procedures, but was stopped early due to futility, as the chronic seton group showed a significantly higher rate of fistula re-intervention compared to both the anti-TNF therapy and the surgical closure after anti-TNF therapy groups (74% *vs* 42% *vs* 23%, respectively, $P = 0.02$). Serious adverse events were numerically higher in the anti-TNF group than the chronic seton group (36% *vs* 21%), but the nature of the events and the rate of infection are not described. Overall, these would suggest that early anti-TNF therapy following seton insertion can be utilized for perianal Crohn's disease.

WHAT SHOULD BE THE TARGET OF TREATMENT?

There has been work to establish a consensus on the most appropriate treatment targets in inflammatory bowel disease, given the variability in treatment outcomes that has been reported in clinical trials, and the fact that treatment goals have evolved and become more stringent with the availability of an increasing number of efficacious therapies. A core outcome set in perianal disease was developed by consensus collaboration between key stakeholders in the United Kingdom following a systematic review of the literature and patient interview by a three-round Delphi process[20]. This identified 3 patient-reported outcomes (incontinence, quality of life and patient priorities) and five clinical-reported outcomes of perianal disease (activity, new abscesses or sepsis, new or recurrent fistula, unplanned surgery and fecal diversion).

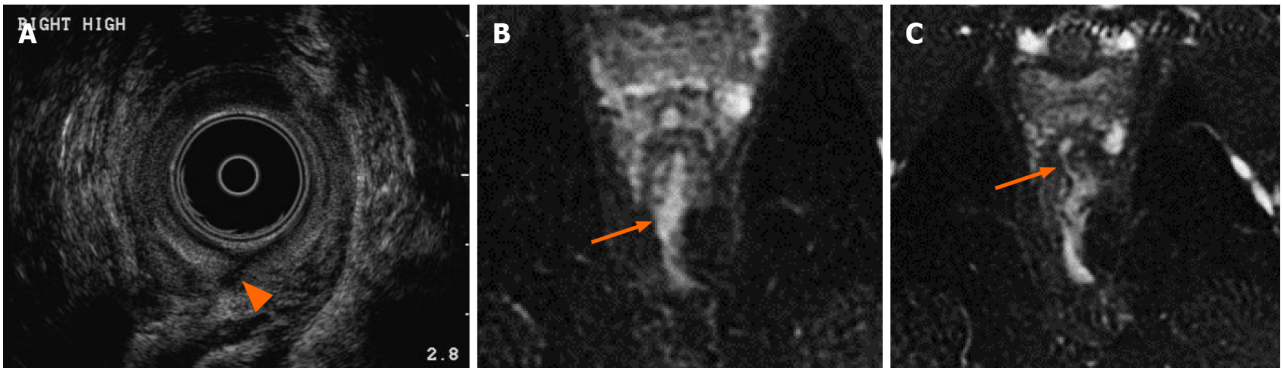


Figure 1 A 37-year-old-man with history of Crohn's disease. A: Transrectal ultrasound identifies a defect in the internal anal sphincter posteriorly (arrowhead); B: Axial T2 weighted, fat saturated image of the anal canal obtained 6 d later more clearly show a transsphincteric perianal fistula arising at 6 o'clock with a fluid filled sinus tract extending posteriorly to exit at the skin surface; C: Magnetic resonance imaging (MRI) image also demonstrates an additional transsphincteric fistula which arises at the 1 o'clock position extending anteriorly and communicating with a complex branching tract which also exited to the skin surface (not shown). MRI provides comprehensive multiplanar imaging of fistulizing disease, allowing visualization of the full extent of disease including supralevator disease and more complete classification of branching or complex fistulas. Additionally, post contrast sequences allow differentiation between fluid containing tracts from granulation tissue seen following healing.

Fistula response on MRI pelvis during follow-up was considered an optional outcome, partially related to issues with the cost and accessibility of MRI. This contrasts with the STRIDE guidelines for luminal inflammatory bowel disease, where endoscopic remission is the agreed target of medical therapy in both Crohn's disease and ulcerative colitis[21,22]. MRI provides a potential advantage over clinical evaluation for tract closure, as it has been shown that internal fistula tracts can persist despite closure of the external opening[23].

Studies evaluating MRI healing and clinical symptoms suggest that MRI healing can lag significantly behind clinical remission, with radiographic findings persisting for up to 12 months after clinical remission[24]. Additionally, many studies of long-term outcomes following treatment with infliximab based on MRI findings have not identified any MRI parameters that accurately distinguish individuals with clinical features of active disease compared to those who achieved clinical remission[25-27]. One observational study suggested that the disappearance of T2 hyperenhancement and absence of enhancement strongly correlated with clinical remission in patients who had an MRI at 12 months after treatment[28]. Many of these studies have used 1.5T MRI machines, which may provide a lower quality image than more modern machines, and there is a lack of study into newer machines, such as 3T machines and evaluation of MRI parameters. Finally, interpretation of perianal protocol MRI is experience-dependent, and outside large centers, there may not be familiarity with the correct imaging protocol and interpretation of these examinations.

Overall, this would suggest that clinical remission remains the main target in treatment of anti-TNF therapy in perianal disease; however, assessment with an MRI pelvis at baseline and during follow-up may provide further information to help guide treatment.

WHAT CAN BE DONE TO OPTIMIZE RESPONSE WHILE ON ANTI-TNF THERAPY FOR PERIANAL CROHN'S DISEASE?

Concomitant medications

Antibiotics: Three trials have evaluated the use of antibiotic therapy, primarily with ciprofloxacin, in combination with anti-TNF treatment for perianal Crohn's disease. All have shown a trend toward improved fistula response rates at 18 weeks and 24 weeks, although none of these results were statistically significant by the end of the study period[29-31]. One of these trials comparing adalimumab with ciprofloxacin to adalimumab with placebo showed an initial significant difference at week 12, with 65% achieving closure of all perianal fistulas on adalimumab with ciprofloxacin compared to 33% on adalimumab with placebo, but this difference was not sustained by the end of follow-up, despite remaining 15% higher in the adalimumab with ciprofloxacin group (62% *vs* 47%, respectively). These studies may have been underpowered to show a significant difference between the groups, and a meta-

analysis suggested that the induction of fistula response (relative risk, 1.58) and remission (relative risk, 1.94) is significantly superior with antibiotics in combination with infliximab compared to anti-TNF therapy alone[32]. The use of antibiotics in this setting, therefore, has been advised by experts, particularly during initial treatment of perianal disease with anti-TNF therapy[33,34].

Immunomodulator therapy: There have not been any dedicated randomized trials specifically comparing use of anti-TNF therapy in combination with immunomodulator therapy to anti-TNF monotherapy. A secondary analysis of two of the placebo-controlled randomized studies comparing infliximab and placebo for perianal Crohn's disease compared anti-TNF therapy in combination with immunomodulators and anti-TNF monotherapy, and did not show a difference in fistula outcomes with induction or maintenance treatment[12,35]. Similar findings were also noted in observational studies comparing combination therapy to infliximab monotherapy[36,37]. It should be noted, however, that patients in these studies had previously failed to achieve a response with immunomodulator therapy prior to initiation of anti-TNF therapy, and some observational studies have shown a significantly higher rate of fistula closure amongst patients on combination therapy compared to anti-TNF monotherapy[38]. None of the aforementioned studies evaluated the use of anti-TNF therapeutic drug monitoring, so it is not clear whether any benefit is related to the effect of the immunomodulator on anti-TNF levels or independent of this. Given the known effects of immunomodulators of reducing immunogenicity and therefore increasing levels of infliximab, and the fact that higher anti-TNF drug levels have been associated with greater response in perianal Crohn's disease (see the below section on 'Therapeutic drug monitoring'), serious consideration should be given to commencing an immunomodulator with anti-TNF therapy, particularly if proactive therapeutic drug monitoring is not being utilized.

Therapeutic drug monitoring

Multiple retrospective and observational studies have identified an association between higher infliximab drug levels and a greater rate of fistula response, healing and closure[39-43]. While the cut-off infliximab level has varied between studies, possibly related to the assay used and end points assessed, it appears that the target level is higher than what is conventionally used to control luminal Crohn's disease (Table 1). Davidov *et al*[44] performed infliximab levels during induction therapy for patients with perianal Crohn's disease and found that those who had a clinical response to treatment by week 14 had significantly higher median infliximab drug levels beginning at week 2 (20 μg/mL *vs* 5.6 μg/mL), and these remained higher than non-responders at weeks 6 (13.3 μg/mL *vs* 2.55 μg/mL) and 14 (4.1 μg/mL *vs* 0.14 μg/mL). Yarur *et al*[39] similarly found significantly higher trough infliximab levels in a cross-sectional study of individuals with perianal Crohn's disease on long-term infliximab who achieved fistula healing compared to those who did not achieve healing (median, 15.8 mcg/mL *vs* 4.4 mcg/mL, $P < 0.001$). This study also found that the odds of achieving fistula healing were over 8 times greater in individuals who underwent dose escalation of infliximab. Intuitively this would suggest that monitoring anti-TNF drug levels proactively and performing dose adjustments in individuals with lower levels may improve fistula healing rates, although this has not been prospectively evaluated.

Duration of anti-TNF therapy and discontinuation

De-escalation of medical therapy in inflammatory bowel disease involves the deliberate reduction in dose or cessation of a treatment once the treatment target has been achieved. A key component of de-escalation of therapy is establishing a treatment target that has good correlation with better long-term outcomes. In luminal Crohn's disease, it has been established that endoscopic remission correlates better with the long-term clinical course of inflammatory bowel disease than symptomatic remission [45]. However, given that treatment of perianal disease remains primarily targeted at symptom-based remission, de-escalation of therapy may be more difficult to complete successfully.

The ACCENT II study evaluated the need for ongoing infliximab therapy in patients with perianal Crohn's disease by comparing the proportion of individuals who lost response to maintenance therapy with either placebo or ongoing infliximab in all individuals who achieved a clinical response to infliximab induction therapy[35]. After 52 wk, 42% of patients had lost response in the infliximab maintenance group compared to 62% in the placebo group ($P < 0.001$). Other retrospective studies have also found that over half of patients who cease anti-TNF therapy will have a clinical

Table 1 Table of trials evaluating ideal cut off value for infliximab and adalimumab concentrations

Ref.	Population (adults or children)	Number	Study design	Follow up or time on therapy	Outcomes	Median level	Median level
Trial: Infliximab							
Plevris *et al* [43]	Adult	29	Retrospective single center cross sectional	2.6 yr	Fistula healing; Fistula closure	8.1; 8.2	3.2; 3.2
Strik *et al*[40]	Adult	47	Retrospective single centercross sectional	3.5 yr	Fistula closure	6.0	2.3
Davidov *et al* [44]	Adult	36	Retrospective observational two centers	Week 2; Week 6; Week 14	Decrease or cessation of fistula drainage at week 14	20; 13.3; 4.1	5.6; 2.55; 0.14
Zhu *et al*[69]	Adult	157	Retrospective single center	Week 30; Week 78; Week 116	Radiological remission (absence of high-signal tracks on fat-saturated T2-weighted sequences)	3.5; 2.85; 2.84	1.9; 1.63; 0.7
El-Matary *et al*[42]	Pediatric	85	Prospective observational 12 centers	Week 14	Clinical fistula healing at week 24	12.7	5.4
Yarur *et al* [39]	Adult	117	Retrospective, cross sectional, 2 centers	29 wk	Fistula healing	15.8	4.4
Trial: Adalimumab							
Plevris *et al* [43]	Adult	35	Retrospective single center cross sectional	1.7 yr	Fistula healing; Fistula closure	14.8; 12.6	5.7; 2.7
Strik *et al*[40]	Adult	19	Retrospective single center cross sectional	3.5 yr	Fistula closure	7.4	4.8
Ruemmele *et al*[41]	Pediatric	36	Randomized control trial	Week 16; Week 52	Clinical fistula closure	7.4; 7.5	6.4; 5.6

relapse with long-term follow-up, so stopping treatment either due to clinical remission or for other reasons is associated with a high risk of relapse[46-48].

Radiological healing is a more stringent treatment target in perianal Crohn's disease, and whether anti-TNF therapy can be stopped after this end point is achieved has not been well studied. A prospective observational study identified 12 patients who were treated with infliximab alone or in combination with a thiopurine who achieved radiological healing[24]. Amongst this group, 5 remained on infliximab therapy and 7 stopped treatments either due to intolerance, treatment de-escalation or switched therapies. There was a trend toward greater rates of radiological healing amongst individuals who remained on infliximab as all maintained radiological remission, while 3/7 in the group that stopped infliximab maintained radiological remission (P = 0.08). Another observational study found that 6/9 (67%) of patients who had achieved radiological healing and stopped maintenance anti-TNF therapy had fistula recurrence[49]. This data would support the use of ongoing anti-TNF therapy in patients who achieve clinical remission of perianal fistulae.

WHAT OTHER THERAPIES CAN BE USED FOLLOWING ANTI-TNF FAILURE?

Vedolizumab

No dedicated randomized controlled trials have assessed the efficacy of vedolizumab for perianal Crohn's disease, and clinical data have shown limited benefit. A post hoc analysis of the GEMINI 2 study compared the efficacy of maintenance vedolizumab to placebo in achieving fistula closure at weeks 14 and 52 following therapy with vedolizumab amongst patients with at least 1 externally draining fistula related to Crohn's disease[50]. Over 40% of patients in each group had previously been treated with anti-TNF therapy. The study identified a numerical difference in fistula closure rates in favor of vedolizumab at week 14 (28% *vs* 11%) and 52 (31% *vs* 11%), although these did not reach statistical significance, and no difference was noted between maintenance therapy given every 4 wk or 8 wk. The ENTERPRISE study was a

randomized trial comparing two intravenous regimens of vedolizumab for treatment of perianal Crohn's disease, with 78% of patients having previously failed anti-TNF therapy[51]. Patients either received standard induction vedolizumab of 300 mg at weeks 0, 2 and 6 then at weeks 14 and 22, while the other group received the same regimen plus a week 10 dose. There was no placebo group in the study and 92% of patients had a seton inserted at baseline. They found that by week 30, 54% of the group had a clinical response to treatment, while 43% had complete closure of fistulae and there was no significant difference between the groups. A nationwide cohort study of 151 patients with perianal Crohn's disease (99% of patients having previously been treated with at least one anti-TNF) who were treated with vedolizumab showed that 23% of patients achieved clinical success (no draining fistula at clinical examination) after 6 mo of treatment, while 67% of patients stopped vedolizumab by 30 wk of treatment due to uncontrolled perianal or luminal disease[52]. Additionally, 31% of patients with perianal disease who had no clinical symptoms at the time of initiation of vedolizumab developed symptoms after commencing therapy. Concomitant immunomodulator therapy was used in 44% of patients in this cohort and this was not a predictor of the success of therapy. Based on these data it appears that there is insufficient evidence to support the widespread use of vedolizumab following anti-TNF failure in perianal Crohn's disease, and further dedicated studies are needed.

Ustekinumab

Available data have provided some support for the use of ustekinumab for perianal disease amongst patients who have trialed or failed anti-TNF therapy. A post hoc analysis of the combined results on fistula healing from the large placebo-controlled trials for ustekinumab in Crohn's disease found that there was a trend toward higher rates of resolution of fistula symptoms by week 8 of ustekinumab therapy compared to placebo (25% *vs* 14%) which had increased to 80% *vs* 46% by week 44[53]. Numbers in the latter analysis were small, with just 26 patients in both groups, and the results were not statistically significant. These trials included a combination of anti-TNF-naïve and anti-TNF-experienced patients, and the results do not distinguish between the groups. A Dutch Nationwide study in which 99% of patients had prior exposure to at least one anti-TNF therapy found that 36% of individuals with perianal fistula at initiation of ustekinumab achieved complete clinical resolution by 24 wk[54]. Further retrospective studies into the use of ustekinumab for perianal Crohn's disease were combined with the aforementioned results in a meta-analysis which found a 56% response rate and 17% remission rate after 52 wk of therapy[55]. Moderate heterogeneity was noted. It appeared that the efficacy of ustekinumab increased between week 8 and week 52. For individuals who are not responding to initial ustekinumab, the use of dose intensification of therapy can result in a clinical response in perianal disease, with one observational study noting 12/24 patients (50%) when escalated to four or six weekly therapy[56]. The limited data available on the use of ustekinumab levels in perianal Crohn's disease do not show an association between serum levels during induction nor maintenance therapy and fistula response at 44 wk[57]. A major limitation of these observational studies is the lack of a comparator being utilized, so while these results suggest ustekinumab may be beneficial in individuals who do not respond to anti-TNF therapy, the size of this effect is not known.

Mesenchymal stem cell therapy

While the exact mechanism that leads to the efficacy of mesenchymal stem cells in the treatment of perianal Crohn's disease is not known, it likely relates to their ability control the local inflammatory response and allow for fistula healing[58]. A placebo-controlled randomized trial found a single dose (120 million cells) of intralesional injection of adipose derived allogeneic cells to the fistula tract in patients with perianal Crohn's disease who were refractory to medical therapy (79% treated with anti-TNF therapy in past 6 mo) achieved combined clinical and radiographic remission in 56% of patients compared to 39% in the placebo group after 12 mo[10,59]. Further studies evaluating the use of both autologous adipose-derived mesenchymal stem cells and bone marrow-derived mesenchymal stem cells have also shown efficacy of these therapies[11,60-62]. While questions remain regarding the optimal dosing and treatment protocol to use, the results suggest treatment to be efficacious and safe even amongst individuals who are not responding to anti-TNF therapy, so this will likely remain an important therapeutic option to consider when it widely available.

Temporary fecal diversion

Reports of the efficacy of a temporary diverting stoma in the pre-biologic era

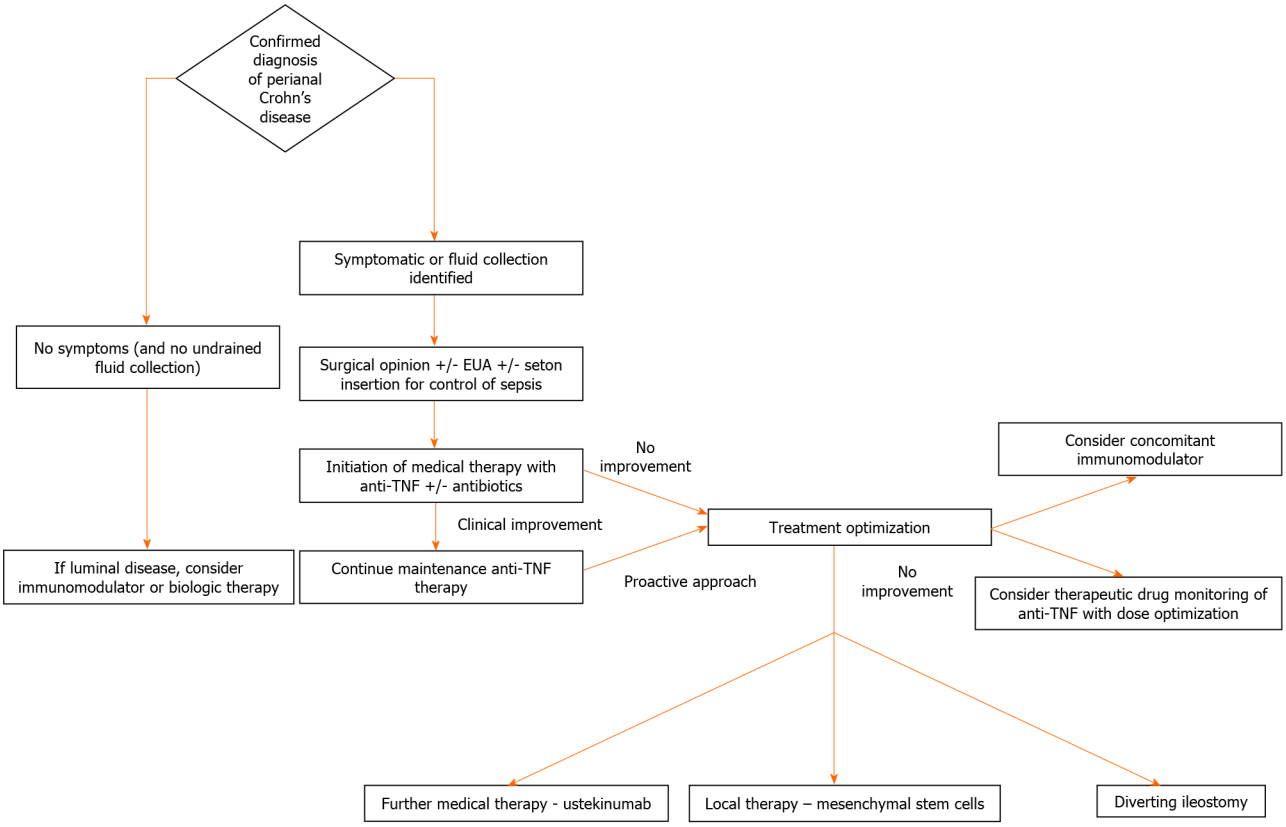


Figure 2 A suggested approach to treatment in perianal Crohn's disease. Anti-TNF: Anti-tumor necrosis factor; EUA: Examination under anesthesia.

suggested it was highly efficacious, with response rates reported to occur in over 80% of individuals in the short term[63,64] and a meta-analysis found response rates in 3 mo to 6 mo in 64% of individuals[65]. However, reports of the use of defunctioning stoma in individuals after failing biologic therapy has shown a lower response rate, with cohort studies suggesting response rates of 46%[66,67]. Rates of successful restoration of bowel continuity also remain low at 18%, and the use of biologic therapy following diverting therapy does not appear to improve the rate of stomal reversal[65,68]. This suggests that while defunctioning surgery remains an option following failure of anti-TNF therapy, the chances of restoration of bowel continuity remain low.

CONCLUSION

Anti-TNF therapy remains the most established therapy for the treatment of perianal Crohn's disease. Emerging evidence would support its early use in treatment, in combination with surgical intervention, to try to minimize complications from perianal disease (Figure 2).

Antibiotics should be considered during induction anti-TNF therapy, but their benefits beyond 24 weeks are not known. While on anti-TNF therapy the use of therapeutic drug monitoring and optimization of anti-TNF levels may improve fistula response rates, but it is likely that higher anti-TNF drug levels are required than are used for the treatment of luminal Crohn's disease. The use of combination therapy with an immunomodulator may be beneficial, particularly in individuals who have not previously failed immunomodulator therapy, although the additional benefit of immunomodulator therapy beyond improving immunogenicity of anti-TNF therapy is not known.

In individuals who fail to respond to anti-TNF therapy for perianal Crohn's disease, ustekinumab may allow for healing of fistula and dose interval shortening of therapy may be of benefit in individuals who are not responding to 8-weekly therapy.

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REVIEW

Incorporating mucosal-associated invariant T cells into the pathogenesis of chronic liver disease

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Abstract

Mucosal-associated invariant T (MAIT) cells have been described in liver and non-liver diseases, and they have been ascribed antimicrobial, immune regulatory, protective, and pathogenic roles. The goals of this review are to describe their biological properties, indicate their involvement in chronic liver disease, and encourage investigations that clarify their actions and therapeutic implications. English abstracts were identified in PubMed by multiple search terms, and bibliographies were developed. MAIT cells are activated by restricted non-peptides of limited diversity and by multiple inflammatory cytokines. Diverse pro-inflammatory, anti-inflammatory, and immune regulatory cytokines are released; infected cells are eliminated; and memory cells emerge. Circulating MAIT cells are hyper-activated, immune exhausted, dysfunctional, and depleted in chronic liver disease. This phenotype lacks disease-specificity, and it does not predict the biological effects. MAIT cells have presumed protective actions in chronic viral hepatitis, alcoholic hepatitis, non-alcoholic fatty liver disease, primary sclerosing cholangitis, and decompensated cirrhosis. They have pathogenic and pro-fibrotic actions in autoimmune hepatitis and mixed actions in primary biliary cholangitis. Local factors in the hepatic microenvironment (cytokines, bile acids, gut-derived bacterial antigens, and metabolic by-products) may modulate their response in individual diseases. Investigational manipulations of function are warranted to establish an association with disease severity and outcome. In conclusion, MAIT cells constitute a disease-nonspecific, immune response to chronic liver inflammation and infection. Their pathological role has been deduced from their deficiencies during active liver disease, and future investigations must clarify this role, link it to outcome, and explore therapeutic interventions.

Key Words: Innate-like lymphocytes; Antimicrobial; Immune regulatory; Pathogenic; Mucosal-associated invariant T cell

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Core Tip: Circulating mucosal-associated invariant T cells are depleted in chronic liver disease, and they have a disease-nonspecific, hyper-activated, immune exhausted, and dysfunctional phenotype. Antimicrobial, immune regulatory, pro-inflammatory, and anti-inflammatory actions are established biological functions of these innate-like lymphocytes, and each function has been invoked to understand the pathogenesis of chronic hepatitis and cholestatic liver disease. Future investigations must establish their pathological role in each form of chronic liver disease, determine the factors that direct function in the hepatic microenvironment, associate deficient functionality with disease severity and outcome, and explore therapeutic manipulations.

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INTRODUCTION

Mucosal-associated invariant T (MAIT) cells are a subset of lymphocytes that engage in innate and adaptive immune responses[1]. MAIT cells react rapidly to pathogens. They can be activated by cytokine stimulation in an antigen-independent manner, and they can eliminate infected or altered cells by releasing pro-apoptotic granzyme B and perforin[2-4]. These features of an innate immune response are complemented by features of an adaptive immune response[1,5]. MAIT cells express a semi-invariant T cell antigen receptor (TCR) that is specific for a limited, but enlarging, array of antigens[6-10]. Furthermore, they can rapidly develop as effector memory cells[11,12].

MAIT cells can recognize riboflavin metabolites or other non-peptide antigens. These antigens are presented by the major histocompatibility complex (MHC) class I-related protein, MR1, which is expressed on antigen-presenting cells (APCs)[13-16]. Antigen-dependent MAIT cell activation can result in chemokine-directed tissue infiltration and release of diverse pro- and anti-inflammatory cytokines[11,12,15,17]. The complex functional phenotype of MAIT cells justifies their inclusion in the family of innate-like lymphocytes[1]. This family includes gamma delta cells (γδ cells)[18-20], natural killer T cells (NKT cells)[21-25], and innate-like B cells (B1 cells, marginal zone B cells, and regulatory B cells)[26-29].

MAIT cells can enhance the adaptive immune response to microbial antigens[2,3]. Bacteria and fungi engaged in riboflavin biosynthesis can trigger the release of pro-inflammatory cytokines and pro-apoptotic molecules that eliminate infected cells[13,14]. Furthermore, the engagement of highly selected microbial antigens with the semi-invariant TCR of the MAIT cells can generate a cytotoxic $CD8^+$ T cell response[2,30]. MAIT cell activation has been ascribed a protective antimicrobial role in several bacterial (*Salmonella typhimurim*, *Mycobacterium tuberculosis*, *Escherichia coli*, and *Klebsiella pneumoniae*)[2,3,31] and viral [dengue virus, influenza virus, and hepatitis C virus (HCV)] infections[32-34].

MAIT cells have now been assessed in chronic hepatitis B[35-39], chronic hepatitis C [40-44], chronic hepatitis D[45], alcoholic liver disease (ALD)[46-48], non-alcoholic fatty liver disease (NAFLD)[49-51], autoimmune hepatitis[52,53], primary biliary cholangitis (PBC)[54,55], primary sclerosing cholangitis (PSC)[56,57], and decompensated cirrhosis[58]. The pathological role of MAIT cells in these diverse forms of chronic liver disease remains unclear. They may be disease-nonspecific perpetrators or facilitators of inflammatory activity[59-62]. MAIT cells may stimulate hepatic fibrosis [52,63] and protect against microbial infection in decompensated cirrhosis[58]. They may modulate the cellular immune response[17,64-66] or limit the immune stimulatory effects of bacterial antigens from the intestinal microbiome[46,67-70].

MAIT cells are emerging as pivotal mediators of chronic liver disease[61,62], and clarification of their pathological role may lead to improved management strategies [71]. The goals of this review are to describe the biological properties of MAIT cells, present evidence of their critical involvement in chronic liver disease, and identify investigational opportunities to clarify their disease-associated roles and therapeutic implications.

METHODOLOGY

English abstracts were identified in PubMed using the primary search words, "MAIT cells", "MAIT cells and chronic liver disease", "MAIT cells and chronic viral hepatitis", MAIT cells and chronic alcoholic liver disease", "MAIT cells and fatty liver disease", and "MAIT cells and autoimmune liver disease". Abstracts judged pertinent to the review were identified; key aspects were recorded; and full-length articles were selected from relevant abstracts. A secondary bibliography was developed from the references cited in the selected full-length articles, and additional PubMed searches were performed to expand the concepts developed in these articles. The discovery process was repeated, and a tertiary bibliography was developed after reviewing selected articles from the secondary bibliography. Eight hundred and fifty-three abstracts and 134 full length articles were reviewed through November 2020.

CANONICAL MAIT CELLS

Defining features

MAIT cells are defined by a semi-variant α-chain within the TCR that is encoded in humans as Vα7.2-Jα33 by the TRAV1-2/TRAJ33 gene[3,7,72,73] (Table 1). The TCR α-chain associates with a constrained number of TCR β-chains. Vβ6 and Vβ20[61,73] are the principal β-chains associated with the TCR of MAIT cells, and they are encoded by the TRBV6 and TRBV20-1 genes in humans[6,8,74] (Figure 1). Together, the α- and β-chains form a TCR that can accommodate a limited number of chemical structures[8,10]. Human MAIT cell TCRs have hypervariable complementarity-determining regions (CDRs) in the TCRα-[75] and TCRβ-[8] chains that have restricted lengths. CDR3β of the TCR Vβ chain is stable between individuals, has a length of 11-14 amino acids, and accommodates 80% of the TCRβ repertoire of MAIT cell antigens[8].

Antigen-presentation to MAIT cells is limited to the class 1b antigen-presenting molecule, MR1[2,7,76]. This restriction of antigen-activation is another defining aspect of MAIT cells (Table 1). The high surface expression of the C-type lectin, CD161[77-79], cytokine receptors for interleukin (IL)-7, IL-12, IL-18, and IL-23[62,77,80,81], and the chemokine receptors, CCR5, CCR6, CCR9, and CXCR6 are other phenotypic features[59,62,77,82] (Figure 1).

Key attributes

Most MAIT cells express the multidrug resistance protein 1 (also called the multidrug *ABCB1* transporter)[11,62,83]. They also possess the nuclear receptor transcription factors, promyelocytic leukemia zinc finger (PLZF) (also known as ZBTB16)[61,84,85], retinoic acid-related orphan receptor gamma t (RORγt)[86], and T-box transcription factor (T-bet)[87-91] (Figure 1). The ATP binding cassette sub-family B member 1 gene (*ABCB1*) renders MAIT cells more resistant to cytotoxic drugs, chemotherapeutic agents, and gut-derived xenobiotics than other lymphocytes[11]. The *ABCB1* transporter does not protect MAIT cells from immunosuppressive drugs such as tacrolimus and mycophenolic acid which are used in the treatment of autoimmune hepatitis[83,92].

The diverse nuclear transcription factors, PLZF, RORγt, and T-bet, are involved in the lineage development of T helper 1 (Th1) cells[87,88,93], memory cells[91], Th17 cells[86], NKT cells[84], and MAIT cells[84,85] (Table 1). PLZP controls the phenotype and functionality of MAIT cells, and it directs the development of an effector memory phenotype[85,94-96]. These memory cells typically have the molecular signature, CD44hiCD95hiCD45RO+CD62Llo[11,12,15,94,97]. They can be activated without prior clonal expansion in response to IL-7[82,98].

PLZP also controls the fate of MAIT cells by activating intracellular caspases and rendering MAIT cells sensitive to apoptotic stimuli[99]. This sensitivity to apoptosis can be counterbalanced by the X-linked inhibitor of apoptosis protein (XIAP)[100]. The counterbalancing effects of PLZP and XIAP may account in part for the variable numbers of MAIT cells detected in the circulation and tissue sites during active inflammation[99].

Activated MAIT cells rapidly secrete interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), IL-17A, and IL-22[11,17,81,101,102]. These cytokines have pro-inflammatory and antiviral effects (Figure 1). Stimulation of human MAIT cells in culture for 7-10 d can induce the robust release of the anti-inflammatory cytokine, IL-13[64,66]. IL-4 and IL-5 are also released but to a lesser extent[65,66]. IL-4 and IL-13 can induce an immunosuppressive phenotype in macrophages[66,103-105], and their

Table 1 Mucosal-associated invariant T cell characteristics and clinical implications

Feature	Characteristics	Clinical implications
Semi-invariant TCR	Semi-invariant α-chain in the TCR[3,7,72]; Canonical Vα7.2-Jα33 α-chain[3,7]; *TRAV1-2/TRAJ33* encodes Vα7.2-Jα33[3]; Vβ6 and Vβ20 most common β-chains [61]; *TRBV6, TRBV20-1* encode Vβ6, Vβ20[8,74]; Restricted length of CDRs[8, 75]; CDR3β key to antigen recognition[10]	Limited number of antigens recognized[8,10]; Antigen diversity still possible[10]
MR1-restricted antigens	Class 1b antigen-presenting molecule[2,7]; Expressed on surface of APC[3]	MR1 limits antigens presented by APCs[10,114]
CD161	High surface expression[77-79]	Shared phenotypic marker with other T cells[78]
Cytokine receptors	IL-7, IL-12, IL-18, IL-23 receptors[80]	Multiple cytokines can activate MAIT cells[80,81]
Chemokine receptors	CCR5, CCR6, CCR9, CXCR6[59,62,77,82]	Chemokine-directed tissue migration[77]
Nuclear transcription factors	PLZF (also known as ZBTB16)[61,84,85]; RORγt[86]; T-bet[87,88,90,91]; ABCB1 [11,62,83]	Control phenotype and functionality[84-88]; Direct development of memory phenotype[91]; Activate caspases and induce apoptosis[99]; Increase resistance to drugs, xenobiotics[11]
Cytokine production	IFN-γ, TNF-α, IL-17A, IL-22[11,17,81,102]; IL-13, IL-4, IL-5 (anti-inflammatory) [64,66]; IL-10 (mainly in adipose tissue)[106]	Pro-inflammatory and antiviral effects[17,81,102]; Anti-inflammatory effects[66,106]; Cross regulation of immune responses[64,65]
Effector phenotype	Granzyme B[4,107,108]; Perforin[4,74,108]	Antimicrobial and pro-apoptotic actions[4]; Eliminates infected or altered cells[4,107,108]
Subsets	Mostly CD8αα cells in liver and blood[101]	More IFN-γ and TNF-α than CD8αβ subset[101]

ABCB1: Multidrug resistance protein 1; APCs: Antigen presenting cells; CDRs: Complementarity-determining regions; IL: Interleukin; IFN-γ: Interferon-gamma; MAIT: Mucosal-associated invariant T cell; MR1: Major histocompatibility complex I-related molecule; PLZF: Promyelocytic leukemia zinc finger; T-bet: T-box transcription factor; TCR: T cells antigen receptor; t RORγt: Retinoic acid-related orphan receptor gamma t; TNF-α: Tumor necrosis factor-alpha.

production in inflammatory liver disease may cross-regulate the pro-inflammatory type 1 immune response[64,65] (Table 1). Little or no IL-10 is produced by activated human MAIT cells in the circulation, but 14% of resident MAIT cells in human adipose tissue produce IL-10[17,106]. Activated MAIT cells also secrete granzyme B and perforin which can have an antimicrobial effect by eliminating infected or altered cells [4,107,108] (Figure 1).

MAIT cell subsets

Most MAIT cells in the liver and peripheral circulation are CD8+ T cells[61,109]. They can be further characterized by the expression of the cell surface glycoproteins CD8α and CD8β[110,111] (Table 1). CD8α can exist as a disulfide-linked homodimer (CD8αα) or as a heterodimer (CD8αβ) on the MAIT cell surface[111]. Most MAIT cells in humans are CD8αα-positive, and most CD8αα-positive T cells in humans are MAIT cells[101] (Figure 1). Other subsets account for less than 10% of the MAIT cell population[101], and they exist as CD8αβ-positive, CD8-CD4- double-negative, and CD4-positive MAIT cells[61,112].

The CD8αα-positive subset produces more IFN-γ and TNF-α than the CD8αβ subset, and it may be more active in inflammatory and immune-mediated diseases [101]. Expansion and maturation of the CD8αα-positive subset of MAIT cells occur after exiting the thymus. The CD8αα-positive MAIT cells are probably driven by antigens encountered in the periphery, including the intestinal microbiome[101,113].

NON-CANONICAL MAIT CELLS AND MAIT-LIKE CELLS

T cell populations other than the typical Vα7.2-Jα33 MAIT cells have a semi-invariant TCR, reactivity to non-peptide antigens presented by a restricted MHC class I-like molecule, and responses that have innate and adaptive immune features. These populations include MAIT cell variants[8,10,74,114], MR1-restricted non-MAIT cells (MR1T cells)[114], and invariant NKT (iNKT) cells[115-119].

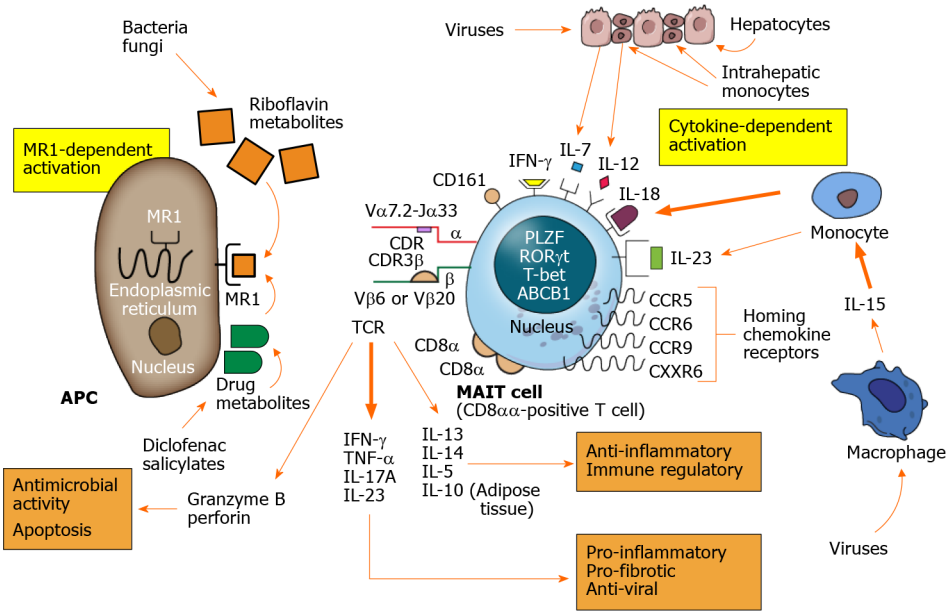


Figure 1 Mucosal-associated invariant T cell activation and actions. Mucosal-associated invariant T (MAIT) cells are activated by MR1-dependent and cytokine-dependent mechanisms. The major histocompatibility complex class I-related protein, MR1, is expressed on the surface of the antigen presenting cell after stimulation. Riboflavin (vitamin B) metabolites synthesized by bacteria and fungi are presented by the MR1 molecule as are select drug metabolites. The T cell antigen receptor of the MAIT cell consists of a semi-invariant alpha (α) chain and restricted beta (β) chain with antigen selectivity influenced by short length complementarity-determining regions. MR1-dependent activation results in MAIT cell production of multiple cytokines as well as granzyme B and perforin. The cytokines can have pro-inflammatory, pro-fibrotic and antiviral effects (lower right panel) and anti-inflammatory and immune regulatory effects (lower right panel). The granzyme B and perforin can have antimicrobial activity and eliminate infected cells by apoptosis (lower left panel). Cytokine-dependent stimulation is activated by phagocytic macrophages and monocytes resident in the liver or circulation and by injured hepatocytes. Multiple cytokines can activate MAIT cells, especially interleukin 18, after virus infection. Chemokine receptors help direct the activated MAIT cells to the site of inflammation, and CXCR6 is the principal chemokine that directs MAIT cells to the liver. MAIT cells contain diverse nuclear transcription factors that influence phenotype and function, especially promyelocytic leukemia zinc finger, retinoic acid- related orphan receptor gamma t, and T-bet. The nucleus also contains the *ABCB1* that affects resistance to gut-derived xenobiotics and certain drugs. The principal subset of activated MAIT cells consists of CD8αα-positive T cells. APC: Antigen presenting cell; TCR: T cell antigen receptor; CDR: Complementarity-determining region; IL: Interleukin; IFN-γ: Interferon-gamma; TNF-α: Tumor necrosis factor-alpha; MAIT: Mucosal-associated invariant T.

MAIT cell variants

MAIT cell variants have semi-invariant TCR α-chains that are encoded by non-classical genes or paired with different TCR β-chains. MAIT cells can have TCR α-chains encoded as Vα7.2-Jα12 by the TRAV1-2/TRAJ12 gene or Vα7.2-Jα20 encoded by the TRAV1-2/TRAJ20 gene[8,73]. These MAIT cell variants have fundamental traits that are identical to the Vα7.2-Jα33 MAIT cell population. They are activated by riboflavin metabolites in a MR1-dependent manner and by cytokine production. They differ only in homing characteristics. The Vα7.2-Jα12 MAIT cells seem to predominate in solid tissues[8]. A MAIT cell variant that is TRAV1-2neg retains the fundamental properties of MAIT cells and warrants inclusion in this variant category[74].

MR1-restricted non-MAIT cells

MR1-restricted T cells (MR1T cells) lack the fundamental characteristics of MAIT cells [114]. MR1T cells have diverse TCRα and TCRβ chains, and they fail to respond to microbial vitamin B metabolites. They produce a wide range of cytokines, and they recognize monocyte-derived dendritic cells as APCs[114]. MR1T cells have specificity for cell-derived antigens that can polarize their function after antigen stimulation [114]. They may thereby influence adaptive immune responses and immune tolerance of self-antigens. MR1T cells represent 1:2500-1:5000 of circulating T cells in healthy individuals, and they can be activated by a wide array of antigens presented in an MR1-binding groove. This MR1-restricted binding groove is larger and less restrictive than the MR1 of MAIT cells[114].

iNKT cells

iNKT cells are similar to MAIT cells in that they have a semi-invariant TCR and react to non-peptide antigens presented by an MHC class I-like molecule[5,81,120]. They express the natural killer (NK) antigen, NK1.1, which is known as CD161 on MAIT cells, and they secrete pro-inflammatory cytokines, including IFN-γ, TNF-α, IL-17, and IL-22[120-122]. iNKT cells differ from MAIT cells in that their TCR α-chain is encoded

as Vα24-Jα18 in humans and paired mainly with the TCR β-chain, Vβ11[120,123]. The antigen-restricted molecule that activates iNKT cells is CD1d (cluster of differentiation 1d) rather than MR1, and the activating antigens are lipid-based, including glycosylceramides (primarily, α-galactosylceramide), glycosphingolipids, and phospholipids [120,124-127]. Furthermore, iNKT cells are rare in the peripheral circulation (0.01%-0.1%)[120,122,128] and liver (0.5%)[120,122,129]. Unlike circulating MAIT cells[17], iNKT cells in the peripheral circulation secrete the anti-inflammatory cytokine, IL-10 [130], and participate in the differentiation of regulatory T cells (Tregs)[23].

MAIT CELL DEMOGRAPHICS

Frequency

MAIT cells constitute 0.1%-10% of the circulating $CD3^+$ T lymphocytes in healthy individuals[61,131], and they are a resident population in the intestine (2%-20%), lung (1%-10%), and liver (10%-40%)[11,61,81,132] (Table 2). Their predominance in the liver [11,43,82,133] and paucity in lymphoid tissue (< 1%)[11] suggest that they are positioned to react with microbial antigens in the portal circulation or metabolic by-products within the liver or biliary circulation[133].

Intrahepatic distribution

MAIT cells are localized mainly in the intrahepatic bile ducts, portal tracts, and sinusoids, and the nature of the liver disease may affect their distribution[55,133] (Table 2). MAIT cells have the ability to migrate based on the expression of tissue-homing chemokine receptors and the location of transmembrane adhesion molecules (integrins)[77]. MAIT cells can be directed to the bile ducts by CCR6, CXCR6, and integrin αEβ7 or to the hepatic sinusoids by CXCR3 and the integrins, LFA-1 (lymphocyte function-associated 1 protein) and VLA-4 (very late antigen 4)[133,134].

Age-related changes

The number of circulating MAIT cells increases from birth to adulthood (ages, 20-40 years) in healthy individuals[135]. Maximum circulating levels are in the third and fourth decades[136] (Table 2). The number of circulating MAIT cells declines after the age of 60 years in association with a gradual increase in MAIT cell apoptosis[135]. It is ten-fold less than in young adulthood after the age of 80 years[136]. The annual decline in the circulating level has been estimated as 3.2% in men and 1.8% in women[131].

Gender differences in the frequency of MAIT cells [131,136] and age-related differences in the phenotype and function of MAIT cells have been described but not established[131,135-137] (Table 2). Advancing age has been associated with an increasing proportion of $CD4^+$ MAIT cells and decreasing proportion of $CD8^+$ MAIT cells in some[131,136,137], but not all[135], ethnic cohorts. The pattern of cytokine production by MAIT cells has also changed to a less inflammatory profile with advancing age in one Asian cohort[131] but not in another[135]. The inconsistent phenotypic and functional age-related alterations may reflect ethnic and environmental factors[135], and they have yet to be ascribed an impact on specific pathological states[136].

MAIT cells are absent in germ-free animals (unlike NKT cells)[7], and their numbers can be reconstituted by intestinal colonization with various microbial organisms[30]. Diverse antigenic encounters during aging may explain the increasing numbers of MAIT cells that are detected in individuals as they enter adulthood[131,135,136].

MAIT CELL ACTIVATION

MAIT cells are activated by MR1-dependent mechanisms that are antigen-mediated and by MR1-independent mechanisms that are mainly cytokine-mediated[71]. Superantigens produced by certain bacteria (mainly, Staphylococcus aureus) can also activate MAIT cells without involvement of MR1[138,139].

MR1-dependent activation

The MR1 molecule is sequestered in the endoplasmic reticulum within the APC[140], and it may be undetectable on the APC surface until antigen exposure[141-146] (Figure 1). The antigen binding groove of the MR1 molecule has two pockets, and it can only bind small molecules[13,147]. The development of murine MAIT cells in the thymus requires exposure to microbial riboflavin metabolites[113]. Most bacteria and

Table 2 Mucosal-associated invariant T cell demographics and clinical implications

Feature	Demographics	Clinical implications
Frequency (based on percentage of $CD3^+$ T cells)	Circulation, 0.1%-10%[11,61,81,131]; Intestine, 2%-20%[11,61,102,132]; Lung, 1%-10%[11,15,61,81]; Liver, 10%-40%[11,43,61,82,133]; Lymph nodes, < 1%[11,61]	Liver is most MAIT cell enriched tissue [61]; MAIT cells can react with microbial antigens and metabolites in portal circulation and in bile[133]
Hepatic distribution	Present in bile ducts, portal tracts, sinusoids[55,133]; Chemokine-directed migrations [77]; CCR6, CXCR6, integrin αEβ7 to bile ducts[77,133]; CXCR3, LFA-1, VLA-4 to sinusoids[77,133]	Nature of the liver disease may direct MAIT cell migration to key site of inflammation[77,133,134]
Age-related changes	Numbers in blood increase up to age 40 yr[135]; Numbers in blood decline after age 60 yr[135]; MAIT cell apoptosis increases with age[135]; Depletion nadir after age 80 yr [136]; Depletion may be faster in men than women[131]; Shift from $CD8^+$ to $CD4^+$ cells with aging[131,137]; May be less pro-inflammatory with aging[131]	Ethnic and environmental factors possible [135]; Uncertain effect on severity and outcome[136]; Consider in design of clinical investigations

LFA-1: Lymphocyte function-associated-1 protein; MAIT: Mucosal-associated invariant T cell; VLA-4: Very late antigen 4.

fungi synthesize riboflavin and generate riboflavin metabolites that can bind to MR1 and activate MAIT cells[30,148].

The principal ligands of MR1 are riboflavin (vitamin B2)-based metabolites designated as ribityllumazines[13] (Table 3). These ligands are characterized by a ribityl tail that can dock with MR1[13,14,74,149]. They are derived from the biosynthetic pathway for riboflavin which is present in most bacteria and yeast[13] (Figure 1). Folic acid (vitamin B9) derivatives, including 6-formylpterin (6-FP), can also be captured by MR1[13], but they lack the ribityl component and are unrecognizable by MAIT cells[74,143]. The synthetic folate derivative, acetyl-6-formylpterin, inhibits MAIT cell function by competing with other bacterial products for MR1 ligation. Its therapeutic value in modulating MAIT cell activity warrants further investigation[143, 150].

Antigen diversity: The antigenic repertoire that binds to MR1 and activates MAIT cells has expanded beyond the ribityllumazines. Transitory neo-antigens have been described that are generated by the interaction of 5-amino-6-d-ribitylaminouracil, an early intermediate in the bacterial synthesis of riboflavin[151], with the metabolic by-products, glyoxal and methylglyoxal[152] (Table 3). Chemically unstable pyrimidine intermediates, 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil (5-OP-RU) and 5-(2-oxoethylideneamino)-6-d-ribitalaminouracil (5-OE-RU), are formed, and these lumazine precursors can activate MAIT cells[9]. The unstable 5-OP-RU and 5-OE-RU molecules have diverse bacterial origins, and they are trapped in the antigen binding groove of the MR1 molecule by a reversible covalent Schiff base[9].

The antigen-binding groove of the MR1 molecule can also accommodate the structurally distinct compounds associated with the metabolism of drugs[153] (Figure 1). Salicylates and diclofenac can generate small molecules that bind with MR1 and exert a stimulatory (salicylates) or inhibitory (diclofenac) effect (Table 3). The findings indicate that diverse ligands outside the riboflavin and folic acid metabolites can be recognized by MAIT cells. They support the prospect that additional antigens will be discovered that modulate MAIT cell function[10,74].

Modulation of MAIT cell response: The MAIT cell response reflects mainly ligand-specific MR1 dependencies and TCR β-chain biases for a particular antigen[148] (Table 3). Different microbial species may produce different riboflavin metabolites and generate a selective MAIT cell response[3] or trigger the memory of a previous microbial exposure[154]. Conformational changes within the CDR3β segment may alter TCR flexibility and modulate antigen recognition within individual MAIT cell populations[143].

MAIT cell activation may also be modulated by the cytokine milieu created by the cells at the site of inflammation (Table 3). IL-7 produced by hepatocytes during inflammation can up-regulate MAIT cell production of Th1 cytokines and IL-17A[82]. Factors that regulate the cell surface expression of MR1 could also influence MAIT cell activation[144]. The non-microbial ligand, 3-([2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl]formamido) propanoic acid, down-regulates cell surface expression of MR1 and prevents antigen recognition[155].

The generation of MAIT cell antigens from bacterial and host-derived metabolic by-products constitute a rapidly responsive mechanism by which to target particular microbial pathogens under prescribed circumstances[9].

Table 3 Mucosal-associated invariant T cell activation and clinical implications

MAIT cell activation	Features	Clinical implications
MR1-dependent stimulation	Adaptive immune response[1,5]; Antigen-triggered MAIT cell activation[8,10,114]; MR1 undetectable before antigen exposure[140,144]; MR1 binds only small non-peptide molecules[147]; Riboflavin metabolites are main MR1 ligands[13]; Ribityllumazines are main riboflavin metabolites[13]; Bacterial and metabolic by-products can activate[9]; Drugs and drug metabolites can bind to MR1[153]	Antigens for presentation restricted[8,10]; Most microbes metabolize riboflavin[148]; Neo-antigens diversify MR1 repertoire[9]; Can develop effector memory cells[11]; Drugs can modulate MR1 signaling[153]; MR1 expression can be inhibited [155]
Modulation of MAIT cell response	Response biased by ligand and TCR β-chain[148]; Riboflavin metabolites differ among microbes[3]; CDR3β rearrangements alter antigen recognition[143]; IL-7 and non-microbial molecules can regulate[155]	Response differs among microbes[154]; TCR plasticity can affect response[143]; Local milieu modulates response[82,155]
Cytokine-dependent stimulation	Innate immune response[1,5]; Activates MAIT cells without TCR ligation[156]; Receptors for IL-7, IL-12, IL-18, IL-23, IFN-γ[81]; IL-18 is main MAIT cell activator[157,158]; IL-18 usually with other mediators[82,157,158]; IL-7, IL-18 produced by hepatocytes[81,158]; IL-1β, IL-18, IL-23 produced by monocytes[81,158]; IL-15 acts on MAIT cells directly and indirectly[158]; Bacteria elicit TLR8-induced cytokines[160]	Initiates rapid antimicrobial response[156]; Response affected by local mediators[81]; Effective against viral infections[32,33,159]; Anti-bacterial monocyte response[160]
Superantigen stimulation	Rapid powerful response to severe infection[138,163]; Bacterial exotoxins activate T cell populations[161]; Foregoes MR1 antigen activation[138,161]; Direct activation by binding to TCR Vβ[71,138,165]; Indirect activation by released IL-12, IL-18[71,138]; Generates robust release of cytokines[138]	MAIT cells are major responders[138]; May result in toxic shock[162]; Causes immune exhaustion[138,139,163]; May exacerbate autoimmune disease[168]; Induces pathogenic autoantibodies[166]

CDR3β: Complementarity-determining region 3-beta; IFN-γ: Interferon-gamma; IL: Interleukin; MAIT: Mucosal-associated invariant T; MR1: Major histocompatibility complex I-related molecule; TCR: T cell antigen receptor; TLR8: Toll-like receptor 8.

Cytokine-dependent activation

MAIT cells can be activated directly by cytokine stimulation in the absence of TCR ligation[156] (Table 3). MAIT cells express cytokine receptors for IL-7, IL-12, IL-18, and IFN-γ, and they can express the receptor for IL-23 after activation[81] (Figure 1). Cytokine activation of MAIT cells is mainly dependent on IL-18 in association with other inflammatory mediators (IL-1β, IL-7, IL-12, IL-15, and the type 1 interferons)[32,33,82,157,158]. These inflammatory mediators are derived from different cell types at the site of inflammation, and they can modulate the MAIT cell reaction in different combinations[81].

Hepatic inflammation can release IL-7 and IL-18 from hepatocytes, and activated monocytes can produce IL-1β, IL-23, and IL-18 after stimulation with IL-15[81,158] (Figure 1). Combinations of IL-1β, IL-7, IL-12, and IL-23 can affect MAIT cell production of IFN-γ and IL-17A[11,81,82]. IL-15 can directly stimulate MAIT cell production of IFN-γ or indirectly induce MAIT activation by stimulating monocyte production of IL-18[81,158].

MAIT cell activation in viral infections (dengue virus, influenza virus, and HCV infections) is dependent on IL-18 production[32]. The antimicrobial protection afforded by MAIT cells against influenza infection is based on the anti-viral activity of IFN-γ. MAIT cells release IFN-γ after stimulation with IL-18 alone[159] or in synergy with IL-12[33,157,160]. Intrahepatic monocytes are activated to produce IL-12 and IL-18 after stimulation of Toll-like receptor 8 (TLR8)[160]. The cytokine milieu at the site of inflammation is a key modulator of MAIT cell activity, and it can tailor or finely tune the MAIT cell response to the disease process.

Superantigen activation

Superantigens are bacterial exotoxins that activate large numbers of T cells without undergoing the conventional route of activation by antigen-presenting MHC class I or class II molecules[70,138,161-163]. The superantigens bind to the lateral surfaces of class II MHC molecules expressed on APCs[164] or to Vβ regions within the TCR of T lymphocytes[70,165]. The massive simultaneous activation of exposed T cells can generate a robust release of cytokines, precipitate a toxic shock syndrome, and result in immune cell exhaustion and anergy[162,166]. Staphlococcus aureus, Streptococcus pyogenes, gram negative bacteria, mycoplasma, and viruses are key producers of superantigens[163].

MAIT cells are major responders to microbial infection and superantigens[138] (Table 3). MAIT cells can be activated directly by binding superantigens to their TCR Vβ region[71,138,139,165]. They can also be activated indirectly by the release of IL-12 and IL-18 from superantigen-activated T cells[71,138]. MAIT cell activation may in

turn induce T cell exhaustion and immunosuppression that prevent adequate control of infection[138].

The impact of MAIT cell activation by superantigens on the occurrence and course of chronic inflammatory or immune-mediated disease is unclear. By activating large numbers of T cells, bacterial superantigens may enhance the proliferation of autoreactive T cells as well as MAIT cells[167,168]. Superantigens may also facilitate production of pathogenic autoantibodies by previously primed B cells[166,169,170]. These consequences could exacerbate an autoimmune disease. Alternatively, superantigens could promote immunosuppression by T cell exhaustion and prevent or ameliorate immune-mediated disease[168]. The actual impact may reflect the timing and intensity of MAIT cell activation[168].

MAIT CELLS IN CHRONIC NON-HEPATIC INFLAMMATORY DISEASES

MAIT cells have been evaluated in diverse chronic non-hepatic inflammatory diseases [15], including systemic lupus erythematosus (SLE)[171], rheumatoid arthritis[171, 172], multiple sclerosis[120,173-176], inflammatory bowel disease[132,177,178], celiac disease[179], and infection with the human immunodeficiency virus (HIV)[109,180]. The findings have commonly demonstrated reduced frequencies of circulating MAIT cells[171,176,178-180], increased numbers of MAIT cells infiltrating the involved tissue (synovium, central nervous system, and ileum)[132,171,173,176], and uncertainties about the pathological role of the infiltration[15,120,181,182]. Furthermore, the studies have been complicated by disparities in the methodology to detect MAIT cells[12,73, 183,184] and difficulties in separating treatment-associated from disease-related findings[15,177,185].

MAIT cell depletion in the circulation has been attributed to tissue migration during inflammation[132,176], apoptosis[99], activation-induced cell death (AICD)[109], exhaustion[186], and concurrent therapy with glucocorticoids[15,177,185]. MAIT cell determinations based on cell surface expression of CD161 have been faulted since chronic inflammation can down-regulate CD161 expression[12,73], and the preferred assay for MAIT cell recognition based on antigen-loaded MR1 tetramers has been used inconsistently[12,73,184,187]. The clinical investigations of MAIT cells in chronic non-hepatic inflammatory diseases provide insights, justifications, comparisons, and caveats that can strengthen and extend the investigations of MAIT cells in chronic liver disease.

MAIT CELLS IN CHRONIC HEPATITIS

The investigations of MAIT cell involvement in chronic hepatitis have been broad, mainly descriptive, and generally consistent with findings demonstrated in other chronic inflammatory diseases. They have positioned MAIT cells at the interface of inflammation and tissue injury, and they have confirmed their lack of disease-specificity. They have also expanded their possible roles as pathogenic, protective, and immune regulatory agents. Furthermore, they have indicated that in some liver diseases MAIT cells have contradictory effects.

MAIT cells may represent a primary, albeit defective, host-directed anti-viral response in chronic viral hepatitis[32,43,45,188]. They may constitute a hyperactive, immune exhausted, and depleted antibacterial defense in alcoholic hepatitis[46,47]. They may be a source of regulatory cytokines that reduce liver injury in NAFLD[51], and they may promote pro-inflammatory responses that enhance tissue injury and hepatic fibrosis in autoimmune hepatitis[52,53]. The MAIT cell investigations in chronic hepatitis provide foundational knowledge that should incentivize further studies and impact on future management strategies.

MAIT cells and chronic viral hepatitis

The number of MAIT cells has been assessed in the blood and liver of patients with chronic hepatitis B[35-39] and chronic hepatitis C[40-44], and the functionality of circulating MAIT cells has been determined in both patient populations[36,39-42].

Reduced frequency of circulating MAIT cells: Five of six studies that have evaluated the frequency of circulating MAIT cells in patients with chronic hepatitis B have found reduced numbers compared to healthy individuals[35-39,45] (Figure 2). In one of these studies, patients with concurrent infection with hepatitis delta virus had more severe

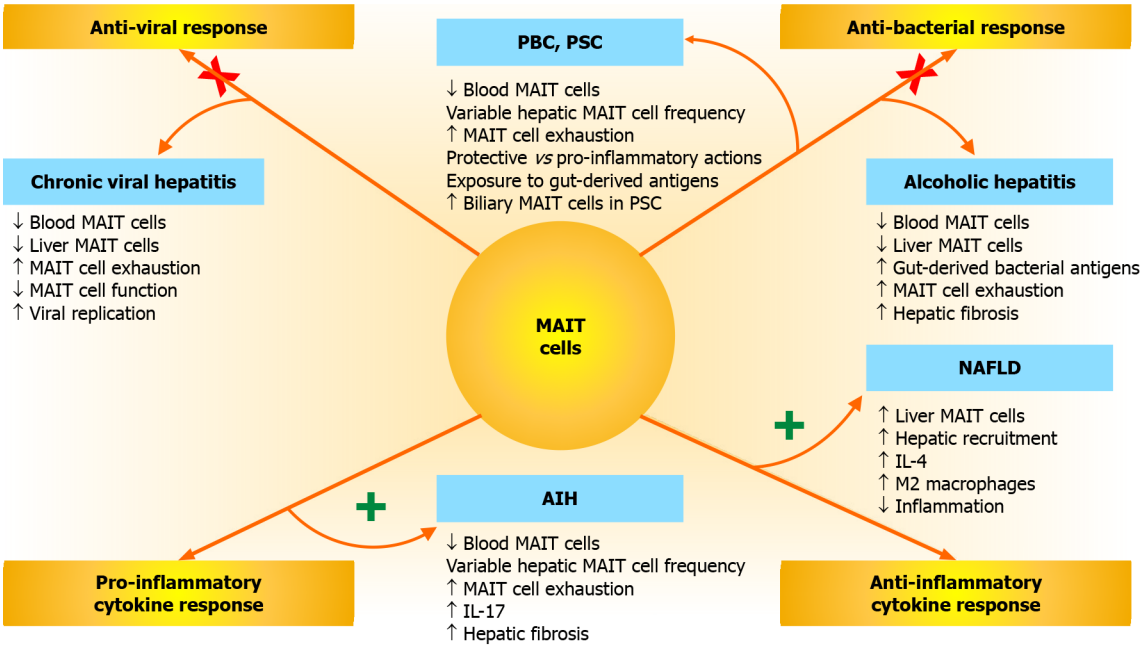


Figure 2 Mucosal-associated invariant T cell responses and associations with chronic liver disease. Mucosal-associated invariant T (MAIT) cells have anti-viral, anti-bacterial, anti-inflammatory, and pro-inflammatory responses that can affect the occurrence, severity, and outcome of diverse chronic liver diseases, including chronic viral hepatitis, alcoholic hepatitis, non-alcoholic fatty liver disease (NAFLD), autoimmune hepatitis (AIH), primary biliary cholangitis (PBC), and primary sclerosing cholangitis (PSC). Impairments in the anti-viral and anti-bacterial responses of MAIT cells (indicated by an "X" across each activation pathway) may promote chronic viral hepatitis, PBC, PSC, and alcoholic hepatitis. Hyperactivity of the anti-inflammatory and pro-inflammatory cytokine responses of MAIT cells (indicated by a "+" next to each activation pathway) may affect NAFLD and AIH. Increased release of interleukin 4 (IL-4) and IL-17 may contribute to the modulation of these responses. MAIT: Mucosal-associated invariant T; NAFLD: Non-alcoholic fatty liver disease; AIH: Autoimmune hepatitis; PBC: Primary biliary cholangitis; PSC: Primary sclerosing cholangitis; IL: Interleukin.

depletion than patients with mono-infection[45] (Table 4). Similar findings of a reduced number of circulating MAIT cells relative to other T cells have been reported in 6 studies that have evaluated this issue in patients with chronic hepatitis C[32,40-44].

Reduced frequency of intrahepatic MAIT cells: The number of intrahepatic MAIT cells was reduced in three of four studies in chronic hepatitis B[35,38,39,45] (Figure 2). Of two studies evaluating the number of intrahepatic MAIT cells in chronic hepatitis C, one disclosed reduced numbers[43] and another failed to associate the marked depletion in blood with an increased accumulation in liver[44] (Table 4). The lack of hepatic accumulation weakens the possibility that hepatic migration of MAIT cells explains the reduced peripheral count. The liver-homing chemokine, CXCR6[189], was also down-regulated on circulating MAIT cells in patients with chronic hepatitis B[39].

Impaired functionality of MAIT cells: Circulating MAIT cells in patients with chronic hepatitis B and chronic hepatitis C have impaired function (Figure 2). This impairment has been manifested mainly by reduced production of granzyme B[36,39,188], IFN-γ [36,39,188], and IFN-α[38] in patients with chronic hepatitis B and by reduced TCR-dependent antigen activation and IFN-γ production in patients with chronic hepatitis C[40,43] (Table 4). In patients with chronic hepatitis B, IFN-γ production has been reduced mainly in patients with serum conjugated bilirubin levels > 10-fold the upper limit of the normal range (ULN). This deficiency has been corrected by TCR-mediated stimulation[39].

In patients with chronic hepatitis C, receptor-mediated, but not cytokine-mediated, activation has been impaired[40,43]. Despite this deficiency, MAIT cells from the liver have had higher levels of activation and cytotoxicity than MAIT cells from the peripheral circulation ($P < 0.0001$). Furthermore, the frequency of MAIT cells in the liver has correlated inversely with histological inflammation ($r = -0.5437$, $P = 0.0006$) and fibrosis ($r = -0.5829$, $P = 0.0002$)[43]. The findings suggest that deficiencies in the number and function of intrahepatic MAIT cells in chronic hepatitis C contribute to the pathological process or are consequences of it.

Table 4 Mucosal-associated invariant T cells in chronic hepatitis and clinical implications

Liver disease	MAIT cell features	Clinical implications
Chronic hepatitis B	Reduced frequency circulating MAIT cells[37,38]; Depletion increased by delta infection[45]; Depleted intrahepatic MAIT cells[38,39,45]; Less granzyme B, IFN-γ, IFN-α release[36,188]; Conjugated bilirubin linked to dysfunction [39]; Increased PD-1 and CTLA-4 on MAIT cells[36]; Exhaustion correlates with HBV DNA level[36]	Chronic activation and exhaustion[36,39]; Less antiviral action[36,39,188]; Increased MAIT cell death[99]; Presumed defective protective role[36]
Chronic hepatitis C	Reduced frequency circulating MAIT cells[41,42]; Depleted intrahepatic MAIT cell[43]; Increased histologic indices reflect depletion[43]; Less TCR-activation and IFN-γ production[40,43]; Increased PD-1 and CTLA-4 on MAIT cells[41]	Hyper-activation and exhaustion[40,41,43]; Increased MAIT cell death[43,61,99]; Antiviral therapy not restorative[40,42,43]; Presumed defective protective role [43]
Alcoholic hepatitis	Reduced frequency in blood and liver[46,47,133]; Decreased granzyme B and IL-17 production[46]; Circulating bacterial products[46,47]; Increased percentage PD-1^{+} MAIT cells[47]; Abundant circulating stimulatory cytokines [47]; Myofibroblasts stimulated and pro-fibrotic[63]	Hyper-activation and dysfunctional[46,47]; Immune exhaustion[46,47]; Impaired intestinal mucosal barrier [46,47]; Increased MAIT cell death[47]; Diminished anti-bacterial function[133]; Presumed defective protective role[46,47]
NAFLD	Circulating MAIT cell frequency decreased[51]; Circulating cells express PD-1 and CD69[51]; Increased intrahepatic MAIT cell frequency[51]; Frequency correlates with NAFLD score[51]; Decreased IFN-γ and TNF-α production [51]; IL-4 induced polarization to M2 macrophages[51]	Activated and immune exhausted[51]; Increased hepatic migration[51]; Recruited by inflammatory activity[51]; Reduced functionality[51]; Promotes anti-inflammatory milieu[51]; Presumed defective protective role[51]
Autoimmune hepatitis	Circulating MAIT cell frequency decreased[52,53]; Reduced granzyme B and IFN-γ secretion[52,53]; Variable intrahepatic frequency[52,53]; Increased IL-17A and HSC stimulation[52]; Increased expression of PD-1 and TIM-3[52]	Activated and immune exhausted[52,53]; Reduced functionality[52,53]; Pro-inflammatory cytokine milieu [52]; Progressive fibrosis[52]; Presumed active pathogenic role[52]

CTLA-4: Cytotoxic T lymphocyte antigen 4; HBV: Hepatitis B virus; HSC: Hepatic stellate cell; IFN-α: Interferon-alpha; IFN-γ: Interferon-gamma; MAIT: Mucosal-associated invariant T; NAFLD: Non-alcoholic fatty liver disease; PD-1: Programmed cell death 1; PBC: Primary biliary cholangitis; PSC: Primary sclerosing cholangitis; TIM-3: T cell immunoglobulin and mucin domain 3; TNF-α: Tumor necrosis factor-alpha.

MAIT cell exhaustion: Circulating MAIT cells in patients with chronic hepatitis B[36,39] and chronic hepatitis C[41] have expressed surface markers indicative of immune exhaustion, especially programmed cell death protein 1 (PD-1), cytotoxic T lymphocyte antigen 4 (CTLA-4), CD39, T cell immunoglobulin mucin protein 3 (TIM-3), CD57, CD38, CD69, and HLA-DR (Table 4). The expression of PD-1 and CTLA-4 and the percentage of MAIT cells expressing these markers have been higher in patients with chronic hepatitis B and viremia than in patients without viremia[36]. Furthermore, the expression of PD-1 and CTLA-4 on MAIT cells has correlated positively with the level of viremia as assessed by plasma levels of hepatitis B virus deoxyribonucleic acid (HBV DNA)[36].

Similar findings have been described in patients with chronic hepatitis C (Table 4). Circulating MAIT cells in patients with chronic hepatitis C have expressed higher levels of surface markers for immune exhaustion (HLA-DR, CD38, PD-1 TIM-3, and CTLA-4) and immunosenescence (CD57) than healthy controls[41]. Chronic immune stimulation and exhaustion could account for MAIT cell dysfunction in chronic viral hepatitis[39] (Figure 2).

Consequences of MAIT cell exhaustion and dysfunction: The consequences of MAIT cell exhaustion and dysfunction in chronic viral hepatitis may include depletion of circulating and intrahepatic MAIT cells and reduced suppression of virus replication [39] (Figure 2). An inverse correlation between the percentage of circulating MAIT cells and the expression of HLA-DR rather than viral load suggests that MAIT cell depletion is a consequence of chronic immune stimulation and exhaustion in chronic hepatitis B[36] (Table 4). MAIT cells have a pro-apoptotic propensity that may contribute to their peripheral depletion in the exhausted state and reduce their accumulation in the liver[43,99].

The positive association of exhausted MAIT cells with plasma HBV-DNA levels in chronic hepatitis B[36] attest to the potential value of MAIT cells as protective agents in chronic viral hepatitis[43]. This association is also supported by the inverse relationship between the number of intrahepatic MAIT cells and the severity of liver inflammation and fibrosis in patients with chronic hepatitis C[43]. The reduced number of intrahepatic MAIT cells in chronic viral hepatitis differs from experiences in non-hepatic chronic inflammatory diseases. In inflammatory bowel disease, rheumatoid arthritis, and multiple sclerosis, MAIT cell numbers are increased in the inflamed tissue consistent with active recruitment[132,171,173,176]. In chronic viral hepatitis,

hepatic recruitment may be less or their intrahepatic destruction more robust[43,61].

Successful antiviral therapy with direct-acting agents does improve the number of intrahepatic MAIT cells[43], but it does not restore the number and function of circulating MAIT cells in patients with chronic hepatitis C[40,42,43] (Table 4). Immune exhaustion is a reversible state in most instances[190-192]. Failure to normalize the circulating MAIT cell population after virus eradication may reflect the low frequency of circulating MAIT cells in healthy individuals, the high density of other immune cells in the circulation, and the long lag time before full restoration[43]. Normalization of the serum conjugated bilirubin level and administration of the mitogen, IL-2, have expanded MAIT cells under experimental conditions in chronic hepatitis B[39]. This observation has not been investigated further.

Conjugated hyperbilirubinemia: The frequencies of circulating and intrahepatic MAIT cells have correlated inversely with the serum conjugated and total bilirubin levels in patients with chronic hepatitis B[39]. Serum conjugated bilirubin levels > 10-fold ULN in chronic hepatitis B have been associated with a reduced number of circulating MAIT cells and a high rate of apoptosis. Surface markers for activation and exhaustion have been present; cytokine production has been variable; and MAIT cell proliferation and expansion have been impaired[39] (Table 4).

Concentrations of conjugated bilirubin equivalent to serum levels > 10-fold ULN have induced apoptosis in MAIT cells obtained from healthy donors, but other direct effects of conjugated bilirubin on MAIT cell function remain unclear[39]. Therapeutic strategies directed at reducing the serum concentration of conjugated bilirubin may be effective because of overall improvement in liver inflammation and function rather than elimination of a prime pathogenic factor[39].

MAIT cells and alcoholic hepatitis

The frequencies of circulating and intrahepatic MAIT cells have been decreased in most studies evaluating patients with alcoholic hepatitis[46-48,63,133] (Figure 2). Furthermore, the residual MAIT cells have been hyper-activated and dysfunctional in a manner similar to patients with chronic viral hepatitis[46,47] (Table 4). MAIT cell expression of transcription factors, RORγt, PLZF, T-bet, and eomesodermin (eomes), has been weak and associated with peripheral MAIT cell depletion and reduced secretion of granzyme B and IL-17[46]. Bacterial products, including endotoxin, D-lactate, and lipopolysaccharide (LPS), have been present in the circulation. Furthermore, macrocyte/monocyte activation by bacterial LPS has been implied by the presence of the soluble receptor for LPS (sCD14)[46,47]. These findings have suggested that bacterial translocation from the intestine is a basis for chronic MAIT cell stimulation in alcoholic hepatitis.

Gut-derived MAIT cell stimulation: Chronic MAIT cell stimulation can impair the expression of nuclear transcription factors that are critical for maintaining antibacterial activity[107]. In alcoholic hepatitis, chronic exposure to gut-derived bacterial products may be a basis for impairing the antibacterial function of MAIT cells and creating a detrimental feedback loop[46] (Table 4). Moreover, fecal extracts from the stools of patients with alcohol-related liver disease have induced MAIT cell depletion in the absence of marked apoptosis or immune exhaustion[46] in a manner suggestive of AICD[46,193].

The presence of gut-derived bacterial markers in the peripheral circulation has also been demonstrated in another study of patients with alcoholic hepatitis[47]. An increased frequency of PD-1-positive MAIT cells has indicated immune exhaustion, and the frequency of exhausted MAIT cells has correlated inversely with the percentage of circulating MAIT cells[47]. Furthermore, there has been a shift in the MAIT cell phenotype from $CD8^+$ to $CD4^+$. The findings suggest that chronic bacterial stimulation contributes to MAIT cell hyper-activation, immune exhaustion, and depletion[47].

Cytokine-mediated MAIT cell stimulation: The hyper-activation of MAIT cells in alcoholic hepatitis could also be ascribed to increased circulating levels of cytokines associated with MAIT cell activation, including IL-7, IL-15, IL-17, IL-18, IL-23, IFN-γ, and TNF-α[47] (Table 4). Most of these cytokines have subsided after 6 mo of alcohol abstinence, but circulating levels of IL-18, IL-23, and IFN-γ have remained elevated for at least 12 mo[47]. Persistence of these cytokines could continue to stimulate and exhaust the MAIT cells. Future investigations must evaluate factors contributing to this sluggish improvement in MAIT cell numbers and function during alcohol abstinence. Persistent permeability of the intestinal mucosal barrier and protracted gut-derived bacterial translocation to the peripheral circulation are key aspects that

require further evaluation.

MAIT cells and hepatic fibrosis: Activated human MAIT cells stimulate the proliferation of hepatic myofibroblasts in co-cultures[63] (Figure 2). The direct contact of activated MAIT cells with myofibroblasts obtained from patients with alcohol-related cirrhosis promotes their proliferation in an MR1-dependent manner (Table 4). The activated MAIT cells release TNF-α which can in turn increase the production of IL-6 and IL-8 by the hepatic myofibroblasts[63]. Under these circumstances, mitigation of MAIT cell activity could have a beneficial effect by reducing the release pro-inflammatory cytokines and the propensity for hepatic fibrosis.

Principal MAIT cell role in alcoholic hepatitis: The principal role of MAIT cells in alcoholic hepatitis appears to be protective. IL-17, which is a product of activated MAIT cells, is pivotal in initiating a cascade of antibacterial responses[194], and activated MAIT cells can eliminate MR1-positive infected cells[133]. MAIT cell dysfunction in alcoholic hepatitis may weaken these defense mechanisms and contribute to a high frequency of bacterial infection (49%)[195] and sepsis (13%)[196]. It may also account for the increased risk of mortality (hazard ratio, 2.33, $P < 0.001$) in severe alcoholic hepatitis[195].

The pathogenic association between MAIT cell dysfunction and adverse outcomes in alcoholic hepatitis remains conjectural, and it awaits studies that establish the protective value of enhanced MAIT cell function. The major therapeutic challenge of investigational MAIT cell manipulation is to promote the antibacterial properties of MAIT cells over their potential pro-inflammatory and pro-fibrotic effects.

MAIT cells and NAFLD

The frequency of circulating MAIT cells is also decreased in patients with NAFLD, and the frequency is inversely correlated with serum concentrations of gamma glutamyl transferase (γ-GGT) and triglycerides[51] (Table 4). Unlike patients with chronic viral hepatitis or alcoholic hepatitis, patients with NAFLD have an increased number of intrahepatic MAIT cells, and this number correlates directly with the NAFLD activity score[51] (Figure 2). Furthermore, the expression of the liver-homing chemokine, CXCR6[189], is increased as is the expression of CCR5 in the circulating MAIT cells [51]. CCR5 promotes MAIT cell migration to areas of hepatic steatosis by interacting with CCL5 (also known as RANTES)[197]. In patients with NAFLD, MAIT cells congregate around degenerating fat-laden hepatocytes in sinusoidal areas[51]. These observations suggest that MAIT cells are actively recruited from the circulation to the liver in response to inflammatory stimuli and hepatic steatosis.

As in chronic viral hepatitis and alcoholic hepatitis, the circulating MAIT cells are activated and immune exhausted. CD69 and PD-1 are expressed on the circulating MAIT cells, and the MAIT cells are functionally altered[51] (Table 4). The production of IFN-γ and TNF-α is decreased, and the release of IL-4 is increased (Figure 2). IL-4 has anti-inflammatory properties which can be manifested in NAFLD as a polarization of Kupffer cells into an M2 phenotype[198].

Macrophages can have a pro-inflammatory M1 phenotype in response to IFN-γ[199] or an anti-inflammatory M2 phenotype stimulated by IL-4[198,199]. A high percentage of monocytes/macrophages co-cultured with activated MAIT cells from patients with NAFLD display the M2 phenotype, and the ratio of M2:M1 macrophages is increased [51] (Figure 2). M2 macrophages can induce the apoptosis of M1 macrophages by a mechanism mediated by IL-10 produced by the M2 macrophages[198]. M2 polarization is a pathway that could reduce inflammatory activity in NAFLD, and it may be influenced by MAIT cells[51].

Free fatty acids increase the surface expression of MR1 in monocyte-derived macrophages[51], and they can also activate macrophages by signaling through TLR4 on the macrophage surface[200]. Free fatty acids could thereby increase macrophage activity and stimulate Kupffer cell expression of MR1. They could also increase TCR-mediated activation of MAIT cells and alter MAIT cell function by immune exhaustion. The net pathological consequence would depend on the balance between MAIT cell production of IL-4, the level of Kupffer cell polarization to the M2 phenotype, the pro-inflammatory effects of free fatty acids, and the extent of MAIT cell exhaustion. Clarification of the sequence and pivotal interactions of these pathological events could identify key targets for therapeutic intervention.

MAIT cells in obesity and diabetes: Obesity and diabetes are conditions that may accompany NAFLD, and MAIT cells can affect these co-morbidities. The frequency of circulating MAIT cells is decreased in obese patients and in obese and non-obese

patients with type 2 diabetes[49] (Table 4). The decreased frequency is accompanied by increased activation (high expression of CD69) of the residual circulating MAIT cells and impaired production of IFN-γ and TNF-α[49]. The frequency of circulating MAIT cells that produce IL-17 is increased with obesity, and the expression of PD-1 is up-regulated, suggesting functional exhaustion[106]. Similar changes have been described in children with type 1 diabetes[50].

Adipose tissue from the omentum of obese patients has a higher frequency of MAIT cells than the peripheral blood (0.59% *vs* 0.06%) consistent with active recruitment, and the percentage of omental MAIT cells producing IL-17 is greater than the percentage in lean persons (26.9% *vs* 7.5%, $P = 0.0009$)[49]. IL-17 induces insulin resistance in adipocytes and hepatocytes[201,202], and the dysfunctional MAIT cells in adipose tissue and peripheral circulation may be a link between obesity and insulin resistance [106].

MAIT cells in human adipose tissue also produce IL-10[106] and IL-4[49]. MAIT cells from obese individuals produce less IL-10 and more IL-17 than MAIT cells from non-obese individuals, and the counterbalance between IL-10 and IL-17 production may modulate insulin resistance[106]. MAIT cells from obese individuals also express less of the anti-apoptotic molecule, B cell lymphoma 2 (Bcl-2)[49]. A shortened survival within the MAIT cell population may be another factor that alters the cytokine milieu.

In a murine model of obesity, MAIT cell frequency has been reduced in blood, epididymal adipose tissue, and ileum compared to lean mice[203]. The MAIT cells in the epididymal adipose tissue of obese mice have produced more IL-17A and TNF-α than in the lean mice, and the MAIT cells in the ileum have produced more IL-17A. The pro-inflammatory cytokine milieu has polarized the macrophages to an M1 phenotype in the epididymal adipose tissue of the obese mice, and it has been associated with an intestinal dysbiosis[203]. Treatment with the folic acid metabolite and TCR-blocking ligand, acetyl-6-formylpterin, decreased IL-17A production in the adipose tissue and ileum, prevented dysbiosis, and improved insulin sensitivity and glucose intolerance[203]. This mouse model of obesity has supported the clinical observations in obese individuals by implicating pro-inflammatory MAIT cells in adipose tissue as a potentially treatable factor contributing to insulin resistance and diabetes risk.

MAIT cell role in NAFLD, obesity, and diabetes: MAIT cells may have a protective role in patients with NAFLD by creating an anti-inflammatory cytokine milieu that polarizes M2 macrophages[51] (Figure 2). This protective role has been supported by animal studies that have demonstrated the development of severe hepatic steatosis[51] or exacerbated diabetes[50] in MAIT cell-deficient mice. MAIT cells may also have a pro-inflammatory effect that contributes to insulin resistance. This possibility has been supported by studies in obese patients[106], diabetic patients[50], and murine models of obesity[203] and diabetes[50]. The clinical and experimental studies in NAFLD, obesity, and diabetes underscore the diversity of MAIT cell activities and the need to better understand the factors that influence these activities within the involved tissue. This understanding is essential before considering targeted therapeutic manipulations.

MAIT cell deficiencies do improve as obesity and diabetes are successfully treated [49,50]. However, the relationship between correction of the MAIT cell deficiency and improvement of the metabolic disease remains conjectural. Bariatric surgery and subsequent weight loss have not normalized MAIT cell frequency or decreased IL-17 production[49]. Furthermore, insulin treatment of type 1 diabetes has not normalized the MAIT cell phenotype in children[50]. The possibility of a genetic predisposition for MAIT cell deficiency or a disease-acquired impairment that persists long-term has not been excluded. Future investigations of MAIT cells in NAFLD and its metabolic co-morbidities will need to define more fully the disease response as a function of MAIT cell activity.

MAIT cells and autoimmune hepatitis

The MAIT cell abnormalities described in untreated autoimmune hepatitis mirror those recognized in other forms of chronic hepatitis. Circulating MAIT cells are activated but depleted[52,53], and they are functionally altered in a manner consistent with immune exhaustion (reduced production of granzyme B and IFN-γ)[52,53] (Figure 2). The number of intrahepatic MAIT cells varies from low[52] to high[53], and the bases for this variation remain uncertain (Table 4). The key insight has been the recognition of MAIT cells as pivotal pro-inflammatory and pro-fibrotic agents.

The frequency of circulating, activated MAIT cells has been significantly higher ($P = 0.009$) in patients with autoimmune hepatitis and advanced hepatic fibrosis (fibrosis

score, F3-F4) than in patients with little or no fibrosis (fibrosis score, F0-F2)[53]. Furthermore, blood levels of IL-17 have been increased in patients with autoimmune hepatitis[204]. IL-17A, which is a product of activated MAIT cells, is a recognized pro-inflammatory and pro-fibrotic cytokine[11,101]. This production has been robust despite features of MAIT cell exhaustion in autoimmune hepatitis[52].

MAIT cells stimulate proliferation of hepatic stellate cells and increase the expression of genes that regulate the production of pro-fibrotic (collagen 1, lysyl oxidase, and tissue inhibitor of metalloproteinase 1) and pro-inflammatory (IL-1β, IL-6, IL-8 and CCL2) molecules[52]. These deleterious actions could help explain progressive hepatic fibrosis in autoimmune hepatitis. They have also been implicated in ALD[63].

MAIT cells have been associated mainly with detrimental pro-inflammatory and pro-fibrotic effects in autoimmune hepatitis[52,53] (Figure 2). Glucocorticoids have decreased the frequency of circulating MAIT cells by 23% in asthmatic patients[177], and they have altered MAIT cell populations in other autoimmune diseases[15,177,185]. Studies that explore the effects of glucocorticoids on MAIT cell numbers and function in patients with autoimmune hepatitis could clarify their pathogenic role and further validate or improve current corticosteroid-based management strategies[92].

MAIT CELLS IN CHRONIC CHOLESTATIC LIVER DISEASE AND DECOMPENSATED CIRRHOSIS

MAIT cells have been evaluated in patients with chronic cholestatic liver disease[54-57] and patients with decompensated cirrhosis[58] (Figure 2). The findings strengthen perceptions already developed in the various forms of chronic hepatitis. MAIT cells are common components of the host response to liver injury independent of etiology or clinical phenotype, and they may be protective, pathogenic, or both protective and pathogenic. The modifiable factors that direct these actions remain unclear and warrant further investigation.

MAIT cells and PBC

MAIT cells in PBC have abnormalities reminiscent of the findings in chronic hepatitis. Circulating MAIT cells are decreased in frequency compared to control subjects[54,55]; intrahepatic MAIT cells are reduced[54] or increased[55] in number; expression of the liver-homing chemokines, CXCR6 and CCR6, are up-regulated; and cytokine production is altered[54] (Table 5). Immune exhaustion has been invoked as a basis for aberrant MAIT cell function[54,55]. Apoptosis, attributable to AICD, has been proposed as a reason for MAIT cell depletion[54,55]. The key insight has been the recognition of MAIT cells as a pivotal immune regulatory population with complex and possibly contradictory actions.

MAIT cells may have a protective effect in PBC. The frequency of circulating MAIT cells has correlated negatively with the serum alkaline phosphatase level, suggesting that the loss of a protective MAIT cell function may permit worsening cholestasis[55] (Table 5). MAIT cell production of IL-17 is increased, and the production of IFN-γ is reduced[55]. IFN-γ is a cytokine that can impair hepatic stellate cell activation[205-207], and the reduced production of IFN-γ by MAIT cells could favor the pro-fibrotic actions of IL-17 in PBC[55,207]. MAIT cells preferentially infiltrate the portal tracts in PBC, and the expression of MR1 is up-regulated in hepatocytes, injured biliary epithelial cells, and inflammatory cells[55]. MR1-mediated stimulation of MAIT cells, possibly by bacterial ligands derived from the intestinal microbiome, could be a protective antimicrobial mechanism in PBC[55].

MAIT cells may also have a pro-inflammatory effect in PBC. The association of MAIT cell activation with elevated serum alanine aminotransferase levels suggests that the MAIT cell response could enhance inflammatory activity[54]. Furthermore, IL-7 stimulates the production of inflammatory cytokines and granzyme B by MAIT cells [55], and IL-7 is increased in the plasma and liver tissue of patients with PBC[55] (Table 5). The bile acid, cholic acid, up-regulates the expression of IL-7 in hepatocyte lines, and the intrahepatic MAIT cells in PBC may develop a pro-inflammatory phenotype[55]. Circulating MAIT cells have reduced expression of receptors for IL-7 (IL-7R) and IL-18 (IL-18R) in PBC, possibly because of immune exhaustion, and it is unclear how this observation might affect the intrahepatic MAIT cell response to IL-7 [54].

Table 5 Mucosal-associated invariant T cells in cholestatic liver disease and decompensated cirrhosis

Liver disease	MAIT cell features	Clinical implications
PBC	Circulating MAIT cells decreased[54,55]; Intrahepatic MAIT cells variable [54,55]; Upregulated liver-homing CXCR6, CCR6[54]; Aberrant MAIT cell function[55]; Depletion associated with increased AP[55]; Low IFN-γ unable to impair HSC activation[55]; Preferential portal tract distribution [55]; Activation associated with increased ALT[54]; Cholic acid-induced hepatocyte IL-7[55]; IL-7-induced pro-inflammatory cytokines[55]; Limited expression of IL-7R and IL-18R[54]	Immune exhaustion[54,55]; Apoptosis-based depletion (AICD)[54,55]; Unable to prevent cholestasis[54,55]; Unable to inhibit hepatic fibrosis[55]; Defective barrier to gut-derived ligands[55]; Pro-inflammatory cytokine milieu [54,55]; UDCA improves but not restorative[54,55]; Presumed defective protective role[54,55]; Presumed active pathogenic role[54,55]
PSC	Circulating MAIT cell frequency reduced[57]; Intrahepatic MAIT cell frequency less[56]; CD69, CD56, PD-1, and CD39 expressed[57]; Impaired response to bacteria[57]; Abundant extrahepatic bile duct MAIT cells[57]	Activated and immune exhausted[57]; Depleted in circulation and liver tissue[56,57]; Less anti-bacterial protection[57]; Abundant migration to bile ducts[57]; Presumed defective protective role[57]
Decompensated cirrhosis	Circulating MAIT cell frequency reduced[58]; High expression of activation markers[58]; MAIT cell frequency increased in ascites[58]; Increased cytokines from peritoneal cells[58]; Increased granzyme B from peritoneal cells[58]; Increased frequency in SBP ascites[58]; Homing chemokine CXCR3 on MAIT cells[58]; Abundant CXCL10 ligand in ascites [58]	Activated and recruited to ascites[58]; Anti-microbial protective response[58]; Protective role of uncertain efficacy[58]

AICD: Activation-induced cell death; ALT: Serum alanine aminotransferase level; AP: Serum alkaline phosphatase level; HSC: Hepatic stellate cells; IFN-γ: Interferon-gamma; IL-7: Interleukin 7; IL-7R: Interleukin 7 receptor; IL-18R: Interleukin 18 receptor; MAIT: Mucosal-associated invariant T; PBC: Primary biliary cholangitis; PD-1: Programmed cell death 1; PSC: Primary sclerosing cholangitis; SBP: Spontaneous bacterial peritonitis; UDCA: Ursodeoxycholic acid.

Therapy with ursodeoxycholic acid (UDCA) for at least 6 mo in 24 patients with PBC has increased the frequency and absolute number of circulating MAIT cells in those patients whose liver tests had improved[55]. The expression of the activation marker, CD25, has decreased; the percentage of apoptotic MAIT cells has diminished; and the frequency of MAIT cells producing IL-17A and granzyme B has decreased. Similar improvements in the frequency and absolute number of MAIT cells have also been demonstrated in 7 patients who had normalized liver enzymes after 6 mo of UDCA therapy[54]. In this series, the frequency of MAIT cells and the expression of IL-7R and IL-18R did not fully recover.

MAIT cells and PSC

Studies of MAIT cells in PSC have been limited, and the findings have been familiar (Figure 2). The frequency of circulating MAIT cells has been dramatically reduced in PSC, and the reduction has been similar to that encountered in patients with inflammatory bowel disease or PBC[57] (Table 5). The MAIT cell depletion has not been associated with particular clinical characteristics, and the MAIT cells have expressed markers of activation (CD69 and CD56) and immune exhaustion (PD-1 and CD39)[57]. The MAIT cell response to bacteria has been reduced, and the cytokine-dependent response has also been impaired[57]. MAIT cells have localized mainly to fibrotic areas in liver samples, and the overall intrahepatic population has been less than in donor or resected liver specimens[56].

The key finding in these studies has been the demonstration of abundant MAIT cells within the biliary tract by brushings obtained at endoscopic retrograde cholangiography[57] (Table 5). The proportion of MAIT cells in the biliary brush samples has been four-fold greater than in matched peripheral blood samples, and the MAIT cell accumulation at the site of inflammation has been consistent with a chronic protective response against intestinal pathogens in the biliary mucosa[57]. Patients with PSC have a high frequency of bacteria in bile cultures, especially with dominant strictures [208-210], manifest intestinal dysbiosis[211,212], and improve clinically after antibiotic therapy[213-216]. These observations have supported the concept that bacterial by-products from the intestinal microbiome stimulates immune-mediated damage to the biliary tree and liver and that MAIT cells have a protective, antimicrobial function in PSC[67,210].

MAIT cells and decompensated cirrhosis

The protective, anti-microbial role of MAIT cells in chronic liver disease has been supported by studies of MAIT cell frequency and function in patients with cirrhosis and hepatic decompensation (Table 5). The frequency of circulating MAIT cells has been reduced in these patients, and the MAIT cells have had an activated phenotype (high levels of HLA-DR, CD25, CD38, CD56)[58]. The frequency of MAIT cells has

been increased in the ascites, and the highly activated peritoneal MAIT cells have produced higher levels of IFN-γ and granzyme B than the MAIT cells in matched blood samples[58].

MAIT cell frequency and total number have also been significantly increased in the ascites of patients with spontaneous bacterial peritonitis compared to patients with uninfected ascites[58] (Table 5). The peritoneal MAIT cells have lacked markers of tissue residency, and they have not been actively proliferating[58]. These features have suggested that the MAIT cells have migrated to the ascites from the circulation. The expression of the homing chemokine, CXCR3, by the peritoneal MAIT cells and the presence of high levels of CXCL10 in the ascites have also supported this conjecture.

MAIT cells in decompensated cirrhosis may have a protective function in uninfected ascites and a critical antimicrobial function in infected ascites. Studies that evaluate interventions to expand and strengthen the MAIT cell population in ascites might improve the control of bacterial infection in patients with decompensated cirrhosis. Investigations in a murine model have already demonstrated that IL-23 combined with the MR1-ligand, 5-OP-RU, can protect against pulmonary infection with Legionella [217], and they encourage similar evaluations in models of advanced liver disease.

INCORPORATING MAIT CELLS INTO THE PATHOGENESIS OF CHRONIC LIVER DISEASE

The incorporation of MAIT cells into the pathogenesis of chronic liver disease requires clarification of the role of MAIT cells in each type of chronic liver disease and demonstration that MAIT cell manipulation can affect disease severity and outcome.

Clarification of the disease-related role of MAIT cells

MAIT cells have been proposed as the new guardians of the liver[17], and patients with chronic viral hepatitis, alcoholic hepatitis, PSC, and decompensated cirrhosis could be protected by the antimicrobial actions of fully functioning MAIT cells[2-4,30,218]. Similarly, patients with NAFLD might be protected by the anti-inflammatory cytokines (IL-4, IL-5, IL-10, and IL-13) generated by MAIT cells in the liver[51] and in adipose tissue[49,106]. Conversely, patients with autoimmune hepatitis could be disadvantaged by abundant MAIT cell production of pro-inflammatory and pro-fibrotic cytokines[52,53], and patients with PBC could be either disadvantaged by MAIT cell production of pro-inflammatory cytokines[55] or benefited by antibacterial actions against products from the intestinal microbiome[55].

The variability of the presumed consequences of MAIT cell depletion or dysfunction in the different liver diseases suggests that local factors in the hepatic microenvironment modulate the nature of the MAIT cell response. These factors may include local inflammatory cues (cytokine milieu)[219], bile acids (cholic acid)[55], gut-derived bacterial products (LSP, endotoxin, antigens)[46,47,69], and metabolic by-products (riboflavin metabolites, glyoxal, methylglyoxal)[9,13,14,151,152].

MAIT cells have different activation thresholds which can modulate their response to non-commensal antigens. Inflammatory signals derived from IL-12, IL-15, or IL-18 elicit robust antiviral (IFN-γ) and antibacterial (granzyme B) responses from MAIT cells[219], whereas direct TCR stimulation induces a brief, less robust release of IFN-γ and TNF-α and a less vigorous effector response[219]. The most potent effector function is elicited when both the inflammatory (cytokine-mediated) and TCR (antigen-mediated) signals are delivered concurrently to the MAIT cells[219]. Conditions at the site of inflammation could modify the activation thresholds for various MAIT cell functions and elicit antimicrobial, anti-inflammatory, or pro-inflammatory actions that are situation-specific[219,220]. Clarification of the conditions that drive the MAIT cell response toward a protective or pathogenic nature are needed to develop targeted, disease-appropriate, therapeutic interventions[220].

Manipulating MAIT cells to assess effects on disease severity and outcome

MAIT cell manipulations are necessary to establish the impact of MAIT cells on disease severity and outcome. Animal studies have demonstrated that genetically manipulated mice without MR-1 and MAIT cells exacerbate experimental NAFLD [51], whereas studies that demonstrate the effect of restoring the MAIT cell population are lacking. These studies would validate a critical pathogenic relationship that is necessary before considering therapeutic manipulations of MAIT cells in clinical trials.

MAIT cells can be up-regulated and down-regulated by several interventions that could help determine their pathogenic role in animal models. MAIT cells are immune exhausted in chronic liver disease, and they may be less responsive to MR1-dependent stimulation[82]. Cytokine-mediated stimulation may be the preferred method of restoring immune exhausted MAIT cells, and recombinant IL-7 has the potential to improve MAIT cell proliferation, cytokine production, effector (granzyme B) function, and MR1-mediated activation[82,107]. Furthermore, the administration of recombinant IL-7 to patients with chronic HIV infection has restored the integrity of the intestinal mucosal barrier[221]. Protection from gut-derived bacterial products by the administration of recombinant IL-7 might have a particular advantage in experimental models of alcoholic hepatitis[46,47], PBC[55], or PSC[57]. IL-15, IL-1β and IL-23 are other cytokines that can activate MAIT cells, but they have less potency than IL-7[82,158,222]. Cytokines of less potency or in various combinations may achieve the desired result without generating opposite or adverse effects.

Drugs and drug-like molecules can also modulate the function of MAIT cells as ligands that bind to MR-1[153]. Diclofenac metabolites can promote MAIT cell activity, and salicylates can inhibit this activity. Drugs have the promise of achieving the MAIT cell response appropriate for the individual disease as an agonist or antagonist of MAIT cell activity. Other therapeutic possibilities are *ex vivo* re-programming of MAIT cell function with select cytokines[82,107], vaccination with IL-23 in combination with 5-OP-RU[217], and re-programming and re-differentiating MAIT cells using induced pluripotent stem cells derived from MAIT cells[223]. Glucocorticoids reduce MAIT cell frequency in certain immune-mediated diseases[15,177,185], and they also may have a role in modulating MAIT cell activity.

Specific molecular interventions that could down-regulate MAIT cell activity are 3-([2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl]formamido) propanoic acid which can impair cell surface expression of MR1 and prevent antigen recognition[155] and acetyl-6-formylpterin, which can inhibit MAIT cell function by competing with other bacterial products for MR1 ligation[143,150]. These molecular interventions have the potential to clarify mechanisms of MR1 expression and function and possibly emerge as experimental agents by which to modulate MAIT cell activity.

MAIT cell manipulation will be an important experimental method to establish the protective and pathogenic importance of MAIT cells in chronic liver disease. This laboratory experience will be essential before considering MAIT cell intervention as a therapeutic strategy in patients.

CONCLUSION

MAIT cells are innate-like T lymphocytes with antimicrobial and immune regulatory properties that are activated, immune exhausted, and dysfunctional in the different types of chronic liver disease. The similarity of findings in diverse liver and non-liver diseases of an infectious or non-infectious nature suggests that MAIT cells are a disease-nonspecific, T cell response to chronic inflammation.

MAIT cells may have a protective function in chronic viral hepatitis, alcoholic hepatitis, NAFLD, and PSC, exert pro-inflammatory and pro-fibrotic actions in autoimmune hepatitis, and have protective and pathogenic effects in PBC. The variable effects of MAIT cells despite their similar clinical phenotype suggests that disease-related factors in the hepatic microenvironment (inflammatory cues, bile acids, gut-derived bacterial products, and metabolic by-products) influence MAIT cell function.

Future investigations must establish the role of MAIT cells as critical determinants of disease severity and outcome in chronic liver disease, identify the local disease-related factors that drive their particular functions, and evaluate interventions that modulate their protective and pathogenic properties in a disease-appropriate manner.

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REVIEW

Artificial intelligence in small intestinal diseases: Application and prospects

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Abstract

The small intestine is located in the middle of the gastrointestinal tract, so small intestinal diseases are more difficult to diagnose than other gastrointestinal diseases. However, with the extensive application of artificial intelligence in the field of small intestinal diseases, with its efficient learning capacities and computational power, artificial intelligence plays an important role in the auxiliary diagnosis and prognosis prediction based on the capsule endoscopy and other examination methods, which improves the accuracy of diagnosis and prediction and reduces the workload of doctors. In this review, a comprehensive retrieval was performed on articles published up to October 2020 from PubMed and other databases. Thereby the application status of artificial intelligence in small intestinal diseases was systematically introduced, and the challenges and prospects in this field were also analyzed.

Key Words: Artificial intelligence; Machine learning; Deep learning; Prognosis prediction; Small intestinal diseases

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Core Tip: Artificial intelligence has been widely used in the management of small intestinal diseases, which has greatly improved the diagnostic efficiency of capsule

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endoscopy and other examination methods, and at the same time, beneficial progression has also been obtained in the prognosis prediction of small intestinal diseases. Although AI still faces risks such as overfitting and black box effects, its stability and efficiency give it great potential in the management of small intestinal diseases. This article reviews the current application status of AI in small intestinal diseases. In addition, challenges and prospects in this field are discussed.

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INTRODUCTION

The small intestine is located in the middle of the gastrointestinal digestive system, with a total length of 5 m to 7 m, including the duodenum, jejunum and ileum, and is the longest organ of the digestive system. Small intestinal diseases (SIDs) mainly include celiac disease (CD), small intestinal Crohn's disease (SICD), primary small intestinal tumor (PSIT), obscure gastrointestinal bleeding and so on. The traditional examination methods include X-ray barium enterography, computed tomography (CT), magnetic resonance imaging (MRI), balloon-assisted enteroscopy, deep enteroscopy and so on. In recent years, the emergence of capsule endoscopy (CE) has brought a revolutionary breakthrough for the diagnosis of SIDs. However, because of the special anatomical position of the small intestine (far away from the oral cavity and anus, overlap and peristalsis), there are still many problems in the diagnosis of SIDs, such as high technical requirements, low positive rate of diagnosis, inaccurate qualitative location of the disease, patient intolerance and so on. In addition, the onset of SIDs is insidious, the specificity of clinical symptoms is low, and the lesion site is not easy to explore, so the clinical diagnosis of SIDs has always been a difficult problem. With the emergence of artificial intelligence (AI) and its wide application in the medical field, it also provides new methods for the whole management process of SIDs and greatly improves the efficiency of SIDs management.

AI is a concept put forward in the 1950s. It is a frontier cross discipline developed on the basis of computer science, neuropsychology, philosophy, linguistics, cybernetics, information theory and so on[1]. The research fields of AI include expert system, machine learning (ML), fuzzy logic, natural language processing and so on. Research methods are also developing continuously, from ML to deep learning and then to convolutional neural network (CNN), promoting the rapid development of research in various fields. The research of AI in the medical field is mainly focused on auxiliary diagnosis. This series of methods of AI has become a hot implementation tool in the field of medical imaging and digestive endoscope[2,3]. Taking the experiment of the breast cancer AI detection system established by Google as an example, the computer-aided diagnosis system based on AI can help doctors reduce the misdiagnosis rate of breast cancer by 5.7%[4]. Researchers at Houston Methodist Hospital also said in their study that they have developed AI software that parses breast X-ray images 30 times faster than ordinary doctors, with an accuracy of 99%[5]. AI is widely used in the study of digestive fields such as gastric cancer[6], colorectal cancer[7], esophageal cancer[8] and so on. AI has also been extensively researched in the field of SIDs, which will be introduced in this paper.

This study used the keywords of "artificial intelligence" and "small intestine" to search the relevant literature in the databases of PubMed, Embase, Web of Science and Cochrane Library up to October 2020. Studies included in our review were required to meet the following inclusion criteria: (1) full-text paper available in English; and (2) studies that associated AI with the small intestinal diseases. We excluded descriptive papers without validation of methods. The application status of AI in SIDs was summarized, and the challenges and prospects in this field were discussed.

AI IN SMALL INTESTINE ANATOMY

Organ segmentation of the small intestine

With the advent of AI, it is possible to perform computer-assisted organ segmentation in CT, MRI, endoscopy and other examination methods and has shown good application potential in the fields like assisted localization of radiotherapy. The following will introduce the research progress of this aspect in the field of the small intestine especially the duodenum (Table 1).

CT: Some studies had used the CNN method to automatically segment duodenum and other abdominal organs from CT images, with clinically acceptable accuracy and efficiency[9,10]. Tong *et al*[11] proposed an end-to-end segmentation network for improving multiorgan segmentation performance using the ML method. The dice similarity coefficient and average surface distance were quantitatively evaluated, and the results confirmed this network had good accuracy and timeliness in the anatomical segmentation of abdominal organs including the duodenum.

MRI: Fu *et al*[12] conducted a retrospective analysis on 3D MR images of 120 patients and proposed a CNN model, which has been verified to accurately segment the abdominal organs including the duodenum and expedite the contouring process for MRI-guided adaptive radiotherapy. Chen *et al*[13] also conducted a similar study, and in their study, the inference process was completed within 1 min, indicating an obvious advantage of timeliness. The length of the small intestine is an important factor in the management of patients with short bowel syndrome. Some scholars designed a special software algorithm to calculate the length of small intestine based on magnetic resonance enterography images in mice. Compared with the measured results of anatomical specimens, the mean absolute difference between the two methods was 1.8 ± 3.8 cm (P = 0.24), and the mean percentage difference was 9.4% ± 6.0%[14].

Endoscopy: In a Japanese study based on GoogLeNet architecture, a CNN diagnostic program was constructed, using 27335 esophagogastroduodenoscopy (EGD) images for the training set and using 17081 EGD images for the independent validation set. The results showed that the CNN has a good effect to classify the anatomical location of EGD images for stomach and duodenum images, with an area under the curve of 0.99[15]. Igarashi *et al*[16] used AlexNet (a deep learning framework) to retrospectively analyze 85246 original images of EGD images in 441 patients with gastric cancer and developed an anatomical organ classifier. The accuracy rates of the training and validation sets were 0.993 and 0.965, respectively.

Diagnosis of small intestinal mucosal lesions

With the emergence of CE in 2000[17], it has revolutionized our understanding of small intestinal mucosa[18-20], enabling doctors to detect small intestinal mucosal erosion, ulcers, vascular disease, bleeding, polyps, parasite and other lesions more efficiently. However, reliable and rapid reading of video is still a challenge, but more and more studies have shown that the combination of AI and CE can greatly improve the efficiency of our evaluation of small intestinal mucosal lesions; the detection accuracy was above 90% in most studies[21-27].

Ulcer: Previous studies have confirmed that applying a CNN system of deep learning to the reading process of CE can reduce the reading time without decreasing the detection rate of erosion and ulcer lesions[28-32].

Angioectasias and bleeding: Intestinal angioectasias cause more than 8% of all gastrointestinal bleeding episodes[33]. Different studies have applied ML, CNN and computer algorithms to the differential diagnosis of intestinal angioectasias and have achieved high sensitivity and specificity[34-39]. AI is also applied to the direct examination of intestinal mucosal bleeding by CE, which can directly calculate the blood content in the digestive tract and infer whether there is active bleeding in the small intestinal mucosa[40-44].

Protruding lesions: There are a variety of small intestinal mucosal protruding lesions. CNN can help doctors describe their shape features, help analyze their nature and distinguish polyps, epithelial tumors, submucosal tumors, *etc.*[34,45,46].

Villous atrophy: Villous atrophy is a defining symptom of some digestive tract diseases such as CD. Some scholars combined AI methods with CE for the detection

Table 1 Applications of artificial intelligence in organ segmentation of the small intestine

Ref.	Diagnostic method	AI technology	Training set	Validating set	Outcomes
Tong *et al*[11]	CT	ML	90 images	-	DSC of duodenum: 69.26%
Kim *et al*[9]	CT	CNN	80 images	40 images	DSC of duodenum: 0.595
Peng *et al*[10]	CT	CNN	43 images	-	DSC of duodenum: 0.61
Fu *et al*[12]	MRI	CNN	100 images	20 images	Dice coefficient of duodenum: 65.50% ± 8.90% Dice coefficient of bowel: 86.60% ± 2.69%
Chen *et al*[13]	MRI	DL	66 images	36 images	DSC of duodenum: 0.80
Takiyama *et al*[15]	EGD	CNN	27335 images	17081 images	AUCs: 0.99
Igarashi *et al*[16]	EGD	ML	49174 images	36072 images	Accuracy (Ts: 0.993, Vs: 0.965)

AI: Artificial intelligence; AUCs: Area under the curves; CNN: Convolutional neural network; CT: Computed Tomography; DL: Deep learning; DSC: Dice similarity coefficient; EGD: Esophagogastroduodenoscopy; ML: Machine learning; MRI: Magnetic resonance imaging; Ts: Training set; Vs: Validating set.

and measurement of villous atrophy and successfully mapped the extent of the diseased small intestine[47].

AI is also used in risk prediction and clinical treatment decisions of small intestinal mucosal lesions. For example, one study used CNN for the risk prediction of acute intestinal bleeding[48], and another study applied CNN to risk prediction and therapeutic tactics selection for duodenal ulcers[49]. Wong *et al*[50] built a ML model, based on data from 22854 patients with gastroduodenal ulcer including six clinical parameters to identify patients at high risk for recurrent ulcer bleeding within 1 year. Gastrointestinal bleeding is a common complication of left ventricular assist device treatment. Axelrad *et al*[51] developed an endoscopic algorithm. Compared with conventional cohorts, the implementation of the algorithm increased endoscopic diagnostic efficiency by 68%, treatment efficiency by 113%, the number of procedures per patient decreased by 27%, the length of hospital stay decreased by 33%, and the estimated cost decreased by 18%.

In addition, the interference of intestinal contents to CE can also be reduced by AI. Combined with support vector machine, Bashar *et al*[52] designed a classifier for separating useless frames that are highly contaminated by turbid fluids, fecal materials and/or residual foods. The accuracy of this classifier was more than 80%. Pietri *et al*[53] developed a computer algorithm to automatically evaluate the demeanor of small intestinal bubbles in CE images. The specificity of this algorithm was 95.79%, the sensitivity was 95.19%, and the calculation time was 0.037 s per frame. It can be used to reduce the interference of bubbles in CE images. Klein *et al*[54] created a computed algorithm based on the pixels in the color bar to score and classify the preparation of the small intestine for CE, and this automatic scoring method has a concordance rate of more than 90% with the assessment of clinicians.

AI IN COMMON SMALL INTESTINAL DISEASES

AI in celiac disease

CD is a complex autoimmune disease. Patients who ingest foods containing gluten will develop an autoimmune response that causes damage to the small intestine. CD is one of the most common chronic digestive diseases, with a prevalence rate of 1% worldwide[55]. Duodenal biopsy is the gold standard for diagnosis[56]. Noninvasive methods such as endoscopy and clinical features analysis are also widely used in diagnosis, but the diagnostic rate of CD is only 15%–20% through current strategies [57]. However, with the increasing application of AI in the diagnosis of CD, the accuracy and efficiency of diagnosis are greatly improved[58] (Table 2).

Previous studies have confirmed that AI-assisted duodenoscope images analysis can greatly improve the diagnostic efficacy of CD, with the accuracy between 80% and 100% and specificity and sensitivity over 80%[59-61]. In the diagnosis of CD, the combination of AI and CE is closer, which can improve the accuracy of diagnosis and

Table 2 Applications of artificial intelligence in celiac disease

Ref.	Diagnostic method	AI technology	Training set	Testing set	Outcomes
Chetcuti *et al*[62]	CE	ML	81 patients	-	Accuracy: 75.3%
Li *et al*[63]	CE	Computer-assisted recognition	Ep: 240, Cp: 220	-	Accuracy: 93.9%
Vicnesh *et al*[64]	CE	Computerized algorithm	21 patients	-	Accuracy: 89.82%
Zhou *et al*[65]	CE	CNN	Ep: 6, Cp: 5	Ep: 5, Cp: 5	Accuracy: 100%
Gadermayr *et al*[59]	EGD	Computer-assisted	290 patients (2835 images)	-	Accuracy: 94%-100%
Das *et al*[67]	Mucosal biopsies	Computer-assisted	Ep: 124, Cp: 137	Ep: 120, Cp: 105	Sen: 90.3%, Spe: 93.5%, AUCs: 96.2%
Wei *et al*[66]	Mucosal biopsies	DL	212 images	-	Accuracy: 95.3%, AUCs > 0.95
Pastore *et al*[70]	Clinical data	Computer-assisted	100 patients	-	Reliability: 0.813
Tenório *et al*[60]	Clinical data	Decision trees, Bayesian inference, k-nearest neighbor algorithm, support vector machines, artificial neural networks	178 patients	38 patients	Accuracy: 80.0%, Sen: 0.78, Spe: 0.80, AUCs: 0.84
Virta *et al*[68]	Micro-CT	Computer-assisted point cloud analysis	81 patients	-	Accuracy: 100%
Sangineto *et al*[69]	Gene expression in PBMCs	ML, random forest algorithm	Ep: 17, Cp: 20	-	Accuracy: 100%

AI: Artificial intelligence; AUCs: Area under the curves; CE: Capsule endoscopy; CNN: Convolutional neural network; Cp: Control group; DL: Deep learning; EGD: Esophagogastroduodenoscopy; Ep: Experimental group; ML: Machine learning; micro-CT: X-ray microtomography; PBMCs: Peripheral blood mononuclear cells; Sen: Sensitivity; Spe: Specificity.

significantly save the diagnosis time[62-65]. AI was also used in the analysis of duodenal mucosa biopsy, which can help with qualitative analysis and play an important role in quantitative analysis[66,67]. At the same time, the application of AI with X-ray images[68], peripheral blood mononuclear cells[69] and clinical features[60, 70] in the diagnosis and classification of CD have also achieved progress.

AI in small intestinal Crohn's disease

Crohn's disease is a chronic nonspecific inflammatory bowel disease that affects the entire gastrointestinal tract, in which 30% of patients are confined to the small intestine, commonly known as small intestinal Crohn's disease[71]. SICD most often involves the distal ileum as well as the jejunum and the digestive tract above and has a higher incidence of intestinal strictures than colonic Crohn's disease[72,73]. The application of AI in the management of SICD is comprehensive, including diagnosis, risk prediction, extra-intestinal manifestation (EIM) prediction and so on (Table 3).

Diagnosis: Lamash *et al*[74,75] used CNN to analyze MRI images and construct an assessment model for SICD. Their model could effectively distinguish active and inactive inflammatory segments, distinguish segments with strictures and segments without strictures and could be used to measure the length of intestinal strictures. Parfenov *et al*[76] used a software diagnostic algorithm to analyze the CE images of 25 SICD patients, preliminarily confirming that CE could be used to diagnose early SICD with intestinal mucosal inflammation. Klang *et al*[77] performed automatic analysis of CE images of 49 SICD patients using a CNN method, achieving diagnostic accuracy of more than 95% and significantly reducing reading time. Yang *et al*[78] also attempted to combine CNN with a microultrasound system for early diagnosis of SICD in mice and achieved good effectiveness in the identification of early inflammation.

Risk prediction of SICD: Taylor *et al*[79] used ML classifiers (elastic network and random forest) to classify small intestine inflammation in asymptomatic first-degree relatives of patients with SICD. They found that genetic variants associated with SICD, family history and fecal calprotectin together identified individuals with presymptomatic intestinal inflammation who are therefore at risk for SICD. Shen *et al*[80] developed a web-based SICD hazard stratification tool. Predicting high-risk populations for SICD based on altered bowel habit, abdominal pain, white blood cell

Table 3 Applications of artificial intelligence in small intestinal Crohn's disease

Ref.	Diagnostic method	AI technology	Training set	Testing set	Outcomes
Yang *et al*[78]	Microultrasound	CNN	43 mice	-	AUCs: 0.8831
Shen *et al*[80]	Clinical data	Computerized algorithm	Ep1: 61, Cp1: 78	Ep2:42, Cp2: 57; Ep3:84, Cp3: 495	AUCs: 0.92
Bottigliengo *et al*[81]	Clinical data	BMLTs (NB, BN, BART)	152 patients	-	AUCs without genetic variables (NB: 0.71, BN: 0.50, BART: 0.76), AUCs with genetic variables (NB: 0.75, BN: 0.67, BART: 0.78)
Taylor *et al*[79]	Clinical data	ML (elastic net and random forest)	480 first-degree relatives	-	AUCs (elastic net): 0.80, AUCs (random forest): 0.87
Menti *et al*[82]	Clinical data	BMLTs	152 patients	-	Accuracy without genetic variables: 82%, accuracy with genetic variables: 89%
Klang *et al*[77]	CE	DL	49 patients (17640 images)	-	AUCs: 0.94-0.99, accuracy: 95.4%-96.7%
Parfenov *et al*[76]	CE	Computerized algorithm	25 patients	-	44% patients confirmed only with the help of AI
Lamash *et al*[74, 75]	MRI	CNN	15 patients	8 patients	Dice coefficients: 75%-97%

AI: Artificial intelligence; AUCs: Area under the curves; BART: Bayes additive return trees; BMLTs: Bayesian machine learning techniques; BN: Bayesian network; CE: Capsule endoscopy; DL: Deep learning; CNN: Convolutional neural network; Cp: Control group; Ep: Experimental group; ML: Machine learning; MRI: Magnetic resonance imaging; NB: Naive Bayes.

count, albumin and platelet count abnormalities allowed clinicians to identify potential SICD earlier.

Risk prediction of EIMs: AI is controversial in the evaluation of the EIMs of SICD. In the study of Bottigliengo *et al*[81], based on Bayesian machine learning technology evaluation combined with genetic factors to predict the occurrence of EIMs in Crohn's disease, it has no advantage over traditional statistical tools. Whereas Menti *et al*[82] used Bayesian machine learning technology to predict the risk of occurrence of EIMs in Crohn's disease, and the prediction accuracy was 82% when considering only clinical factor and 89% combined with genetic factors, which was outperforming other prediction techniques.

AI in primary small intestinal tumor

The incidence of PSIT is about 5% of gastrointestinal tumors and 0.2% of all kinds of tumors[83,84]. The main site of PSIT is the duodenum, followed by the jejunum and ileum[85]. There are a variety of pathological types of malignant PSIT. Adenocarcinoma is the most common pathological type, up to 40%, followed by neuroendocrine tumors (25%), malignant lymphomas (10%-15%) and malignant stromal tumors (9%)[86]. PSIT lacks specific manifestations in the early stage, and they are faced with many problems in the clinic, such as difficult diagnosis, high misdiagnosis rate, nonstandard treatment and so on[87]. AI has been applied in the field of auxiliary diagnosis and prognostic analysis of PSIT, and has an important impact on the management (Table 4).

Diagnosis: Inoue *et al*[88] used CNN to analyze EGD images for the diagnosis of superficial nonampullary duodenal epithelial tumors. The overall diagnosis accuracy of CNN was 94.7%, including 94% for adenomas and 100% for high-grade dysplasias, and it only took 12-31 s for analysis. The method of support vector machine was applied to the automatic analysis of CE images, which greatly improved the accuracy and efficiency of diagnosis[89-92]. In addition, Barbosa *et al*[93] used the CNN to automatically analyze CE images for the diagnosis of PSIT, which also had high sensitivity and specificity, reaching 98.7% and 96.6%, respectively.

Risk stratification and prognosis prediction: In different studies, ML was used to analyze the pathological tissue samples, plasma protein multibiomarker and miRNA markers of patients with small intestinal neuroendocrine tumors[94-96]. Their studies provided some new and effective methods for early diagnosis, treatment strategy selection, prognosis prediction and recurrence risk prediction of small intestinal

Table 4 Applications of artificial intelligence in primary small intestinal tumor

Ref.	Diagnostic method	AI technology	Training set	Testing set	Outcomes
Inoue *et al*[88]	EGD	CNN	531 images	1080 images	Accuracy: 94.7%-100%
Liu *et al*[90]	CE	SVM	89 patients	-	Sen: 97.8%, Spe: 96.7%
Vieira *et al*[89,91]	CE	SVM	29 patients (936 images)	-	This SVM outperforms others by more than 5%
Barbosa *et al*[93]	CE	CNN	Ep: 104, Cp: 100	Ep: 92, Cp: 100	Sen: 98.7%, Spe: 96.6%
Panarelli *et al*[94]	MicroRNA sequencing	ML	84 samples	-	Accuracy (Ts: 98.5%, Vs: 94.4%)
Drozdov *et al*[95]	Gene expression profiling	ML	73 samples	-	Differentiated from normal cells (Sen: 100%, Spe: 92%), metastases prediction (Sen: 100%, Spe: 100%)
Kjellman *et al*[96]	Plasma protein multibiomarker	Random forestmodel	Ep:135, Cp: 143	-	AUCs: 0.97
Yan *et al*[97]	CT	Random forestmodel	213 patients	-	AUCs: 0.943

AI: Artificial intelligence; AUCs: Area under the curves; CE: Capsule endoscopy; CNN: Convolutional neural network; CT: Computed tomography; Cp: Control group; EGD: esophagogastroduodenoscopy; Ep: Experimental group; ML: Machine learning; SVM: Support vector machine; Sen: Sensitivity; Spe: Specificity; Ts: Training set; Vs: Validating set.

neuroendocrine tumors. In the study of Yan *et al*[97], random forest models were performed to evaluate the correlation of risk stratification for small intestinal stromal tumors. Their study suggested multidetector CT texture analysis may become an important comprehensive tool for preoperative risk stratification of small intestinal stromal tumors.

AI in other small intestinal diseases

Small intestinal obstruction: Cheng *et al*[98,99] used CNN to analyze abdominal radiographs to assist in the diagnosis of small intestinal obstruction (SIO). The sensitivity and specificity of the CNN diagnostic system were 83.8% and 68.1%, respectively, based on the training set of 2210 abdominal radiographs. When the training set was expanded to 7768 abdominal radiographs, the diagnostic sensitivity and specificity were increased to 91.4% and 91.9%, respectively. Their study suggests that the accuracy of detection of SIO by CNN improves significantly with the increasing number of training radiographs. Lucas *et al*[100] explored the development of an ML tool for SIO detection based on CT images. They evaluated the accuracy of eye tracking in image centerline annotation of the small intestine as the first step in the development of an ML tool for SIO. Their results showed that the eye tracking-based annotation was accurate and precise enough for application in ML-based small intestinal centerline annotation.

Small intestinal motor dysfunction: Small bowel intestinal dysfunction (SIMD) can occur during the development of many diseases, so the evaluation of small intestinal motor function is an important means for the auxiliary diagnosis and severity evaluation of these diseases. AI is widely used to evaluate small intestinal motor function through CE images. Using intraintestinal manometry as the gold standard for the diagnosis of SIMD, Malagelada *et al*[101] proved that an ML model was reliable to evaluate CE images for the diagnosis of SIMD. Applying this model, they found that 29% of patients with functional intestinal disorders had SIMD, significantly higher than that of the healthy population (3%), confirming the pathophysiological changes in the intestine of functional intestinal disorders[102]. Furthermore, a classification method for classifying functional intestinal disorders according to small intestinal motor function was also proposed[103].

De Iorio *et al*[104] also demonstrated that ML can reliably detect reduced intestinal muscle activity and motion by CE images through the method of injecting intestinal muscle inhibition (glucagon) into healthy subjects. Moreover, the study of Seguí *et al*[105] suggested that CNN was also reliable in the description and classification of small intestine motor characteristics with the classification accuracy reaching 96%. Malagelada *et al*[106] also used ML to analyze CE and abdominal MRI images of patients with cystic fibrosis and confirmed that the delay in small bowel and colonic transit times in patients with cystic fibrosis is associated with known endocrine

dysfunction and with SIMD.

Small intestinal ischemia-reperfusion injury: Intraoperative evaluation of intestinal viability in patients with acute intestinal ischemia is a critical factor for surgical decision making. In the pig jejunum experiment, Strand-Amundsen *et al*[107] attempted to apply ML to the analysis of multivariate time-series of bioimpedance sensor data to analyze intestinal viability after intestinal ischemia-reperfusion. The results suggested that the measurement should be made before the onset of reperfusion, and the prediction effect was better when the measurement was repeated continuously during ischemia and reperfusion. The detection accuracy of irreversible damage may be close to 100%.

Enteropathies associated with undernutrition: There is a significant histopathological overlap in duodenal biopsies of enteropathies associated with undernutrition such as environmental enteropathy and CD. Syed *et al*[108] used CNN to establish a histopathological analysis model, which can effectively distinguish environmental enteropathy, CD and normal intestinal mucosa. The detection accuracy was 93.4%, and the false-negative rate was 2.4%.

CHALLANGES AND PROSPECTS

At present, in order to promote the application of AI in the field of SIDs, we still need to solve some problems and challenges: (1) Insufficiency of training sample size. The incidence of most SIDs is not high. For example, the incidence of PSIT is much lower than that of gastric tumors and colorectal tumors, which affects the amount of data in the training set. Measurement errors are easy to occur when the sample size is small [109], and as suggested by the continuity study of Cheng *et al*[98,99], expanding the sample size of the training set can significantly improve the inspection accuracy of AI model; (2) Lack of prospective data. Most of the studies used retrospective data, which have been artificially screened before AI model training and lack prospective studies; (3) Single source of data. Most of the training sets and verification sets used in the study come from single-center data, so it is still necessary to further improve the repeatability and stability of the model through multicenter data. As in Alzheimer's disease research, each single center should be encouraged to share data in anticipation of establishing large-scale and open databases[110]; (4) The interpretation of the results. Due to the inevitable problems of AI, like overfitting of training set data[111] and the "black box" characteristic of the algorithm[112], the accuracy and interpretation of the AI model are inconsistent, which may have a negative impact on clinical application[113]. More intensive basic research and extensive verification are needed to improve this deficiency; (5) Ethical and legal issues. Can we trust the results of AI? Once the AI diagnosis and treatment prediction fails, it will give rise to a series of social, ethical and legal problems[114,115]. It is necessary to combine human supervision with AI tools more reliably; and (6) AI has been widely researched in various fields. We should attach importance to learning experience from different research fields and try to carry out related research in the field of SIDs, so as to promote the continuous progress of AI research in the field of SIDs.

CONCLUSION

The advantages of AI in the diagnosis and prognosis analysis of SIDs have been increasingly recognized, and the high accuracy and efficiency of AI detection greatly reduce the workload of doctors. Although there are still various challenges in the application of AI, the potential of AI in improving the management efficiency of diseases cannot be ignored. Clinicians should work together with experts in various fields to promote the development of AI in SIDs.

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REVIEW

Impact of the COVID-19 pandemic on inflammatory bowel disease patients: A review of the current evidence

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Abstract

Since the initial coronavirus disease 2019 (COVID-19) outbreak in China in December 2019, the infection has now become the biggest medical issue of modern medicine. Two major contributors that amplified the impact of the disease and subsequently increased the burden on health care systems were high mortality among patients with multiple co-morbidities and overcapacity of intensive care units. Within the gastroenterology-related community, particular concern was raised with respect to patients with inflammatory bowel disease (IBD), as those patients are prone to opportunistic infections mainly owing to their immunosuppressive-based therapies. Hence, we sought to summarize current knowledge regarding COVID-19 infection in patients with IBD. Overall, it seems that IBD is not a comorbidity that poses an increased risk for COVID-19 acquisition, except in patients treated with 5-aminosalicylates. Furthermore, outcomes of the infected patients are largely dependent on therapeutic modality by which they are treated, as some worsen the clinical course of COVID-19 infection, whereas others seem to dampen the detrimental effects of COVID-19. Finally, we discussed the present and the future impact of COVID-19 pandemic and concomitantly increased health care burden on IBD-management.

Key Words: COVID-19; SARS-CoV-2; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis

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Core Tip: Coronavirus disease 2019 (COVID-19) is the biggest medical issue of the 21st century so far. Within the gastroenterology-related community, COVID-19 is a concern in patients with inflammatory bowel disease (IBD), as those patients are prone to opportunistic infections owing to their immunosuppressive-based therapies. Hence, in this review, we summarized currently available data and concluded that patients with IBD are not at a higher risk for COVID-19 development, unless treated with 5-aminosalicylates, and that the outcomes of infected patients depend on their respective therapeutic modalities. Finally, we discussed the impact of the COVID-19 pandemic and the concomitantly increased health care burden on IBD-management.

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INTRODUCTION

With the immense burden that coronavirus disease 2019 (COVID-19) posited on health care systems and the global economy in general, the disease is unequivocally the biggest medical concern of the 21st century so far[1]. Globally, by January 2021, there have been over 84 million confirmed cases of COVID-19, with more than 1.8 million deaths reported by the World Health Organization (WHO)[2]. The causative agent of the pandemic, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), belongs to the family of *Coronaviridae*, a group of viruses which have already been associated with epidemics in the early 2000s, *i.e.*, severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS)[3,4]. Similar to its related viruses, SARS-CoV-2 primary pathologic manifestations occur in the respiratory system[5,6]. Initial clinical presentation is usually characterized by fever, cough, shortness of breath and specific loss of smell and taste, whereas in a smaller, yet significant amount of patients, the disease progresses to severe lung injury, resulting in the need for intensive care support and concomitant tertiary care equipment[7,8]. This raises two principal issues surrounding COVID-19 and explains why it has caused such a huge global impact. The issue first is high mortality in those patients and the other is the overcapacity of intensive care units (ICU), subsequently burdening health care systems. In the early phases of the pandemic, based on initial observations and knowledge about communicable diseases, particular concern was raised with respect to patients who were at high risk of acquiring severe illness. High risk patients mainly consist of the elderly, obese and patients with pre-existing comorbidities, especially those who are immunocompromised and immunosuppressed[9-11].

Therefore, within the gastroenterology-related community consisting of both medical staff and patients, inflammatory bowel disease (IBD) emerged as an important concern, mainly owing to the IBD therapeutic approach as opposed to the disease itself. Although IBD pathophysiology includes immune dysregulation, the available data does not support the notion that patients with IBD are at a higher risk of acquiring communicable diseases[12-14]. Nevertheless, the IBD therapeutic approach is mainly based on a palette of immunosuppressants, medications whose role in promoting opportunistic infections has been well-established[15-18]. Hence, major organizations instantly provided recommendations for the management of patients with IBD[19-21]. However, knowledge regarding SARS-CoV-2 evolves on a daily basis, resulting in updates to recommendations in order to reach an optimal approach for patients with IBD. In this review, we sought to address the main concerns regarding the relationship between of IBD and COVID-19. Specifically, we summarized the current data and tried to elucidate whether IBD is associated with a higher risk of COVID-19 infection, whether infected patients have worse outcomes than the general population and finally, we discussed how the COVID-19 pandemic and concomitantly increased health care burden influenced IBD-management.

THE RISK OF COVID-19 INFECTION AMONG PATIENTS WITH IBD

Early in the pandemic, a major concern among gastroenterologists was about the occurrence of COVID-19 among patients with IBD for several reasons. SARS-CoV-2 binds to targeted cells *via* angiotensin-converting enzyme 2 (ACE2), a protein constitutively expressed by epithelial cells of the blood vessels, lung, kidney and especially the intestines, where ACE2 expression is among the highest in humans[22-24]. Moreover, as shown by proteomic tissue analysis, ACE2 gastrointestinal expression is increased in IBD patients, especially among the Crohn's disease (CD) subgroup, where expression is markedly higher than in ulcerative colitis (UC)[25]. ACE2 has been also implicated in the pathophysiology of IBD, having a dual-role: aggravation of colitis *via* the classical renin/angiotensin II/aldosterone pathway and amelioration of colitis *via* the ACE2/MAS-1 receptor pathway[26-29]. Except for ACE2, SARS-CoV-2 pathogenesis depends on the specific "spike" glycoprotein that mediates fusion of the coronavirus envelope with the host cell membrane[30]. This protein is activated through the trypsin-like protease, the transmembrane protease serine 2 (TMPRSS2), the activity of which has been shown to be up-regulated in IBD[31]. Furthermore, in up to 50% of COVID-19 patients, fecal samples were positive for SARS-CoV-2 virus, with more than one-fifth of the samples testing positive even after subjects tested negative from respiratory samples, implicating the fecal route of SARS-CoV-2 transmission[32,33]. This could be even more important, as patients with IBD are more frequently assessed with invasive gastrointestinal procedures such as esophagogastroduodenoscopy and ileocolonoscopy compared with the non-IBD population, subsequently exposing both the patient and the examiner to a higher risk of infection[34]. Finally, the use of IBD immunosuppressive therapies has been associated with an increased risk of infections[13,14]. All of these findings suggest that patients with IBD should be the "perfect" host for SARS-CoV-2 viral infection (Figure 1). However, results from a recent systematic review that comprised 13 cohort studies and 5 single case reports from all around the world suggest that patients with IBD do not seem to have a higher risk of COVID-19 infection with respect to the general population, not even in IBD patients treated with immunosuppressive drugs [35]. Another systematic review and meta-analysis by Singh *et al*[36] concluded similarly and additionally determined that there was no difference in COVID-19 occurrence between IBD subgroups, *i.e.*, between CD and UC. Regarding the IBD therapeutic strategies, Singh *et al*[36] demonstrated that no use of therapeutics was associated with an increased risk of COVID-19 acquisition aside from the use of 5-aminosalicylic acid (5-ASA). In fact, Taxonera *et al*[37] evaluated the age-standardized incidence of COVID-19 in IBD patients, and suggested that COVID-19 incidence might be overestimated in the IBD population. Unfortunately, larger studies did not conduct a comparable evaluation.

Multiple authors struggled to explain the discrepancy between the expected and evidence-based COVID-19 incidence among populations with IBD[36,38]. Firstly, a major determinant to reduced COVID-19 incidence could be the tighter containment of patients with IBD, since people suffering from chronic diseases, especially patients treated with immunosuppressants, were warned by experts to follow strict social distancing measures, known as shielding, from the beginning of the pandemic. Furthermore, ACE2, the above-noted protein that is up-regulated in IBD, has two distinct functional forms. The full-length form of ACE2 possesses an extracellular domain that binds to the SARS-CoV-2 virus and transmembrane domain, which anchors the first domain to the plasma membrane and aids viral entry into the cell[39]. Conversely, the soluble form of ACE2 lacks a transmembrane domain and it is therefore a sort of a decoy receptor for SARS-CoV-2 virus in the blood[40,41]. Notably, the latter form is up-regulated in IBD patients, as a consequence of ACE2 membrane cleavage into the soluble form, in a process regulated by the tumor necrosis factor-alpha (TNF-α) convertase ADAM17 (a disintegrin and metalloproteinase 17), the protease is up-regulated in patients with active IBD[42,43]. Furthermore, although SARS-CoV-2 is detectable in fecal samples and active viral replication in the enterocytes of the small intestine has been reported[33,44], to this day there is no firm evidence to imply that increased SARS-CoV-2 replication in intestines is proportional to intestinal ACE2 expression. This is substantiated by the fact that SARS coronavirus, a SARS-CoV-2 close relative, spreads through the upper respiratory tract (URT) very effectively despite only modest ACE2 expression in the URT[24]. Overall, it seems that SARS-CoV-2 also needs the presence of a co-receptor for host cell infection, similarly to HIV infection[45]. However, the hypothesized co-receptor that synergistically with ACE2 leads to SARS-CoV-2 infection has yet to be determined in future studies. The reasons for the increased risk of COVID-19 acquisition with 5-ASA are still unclear

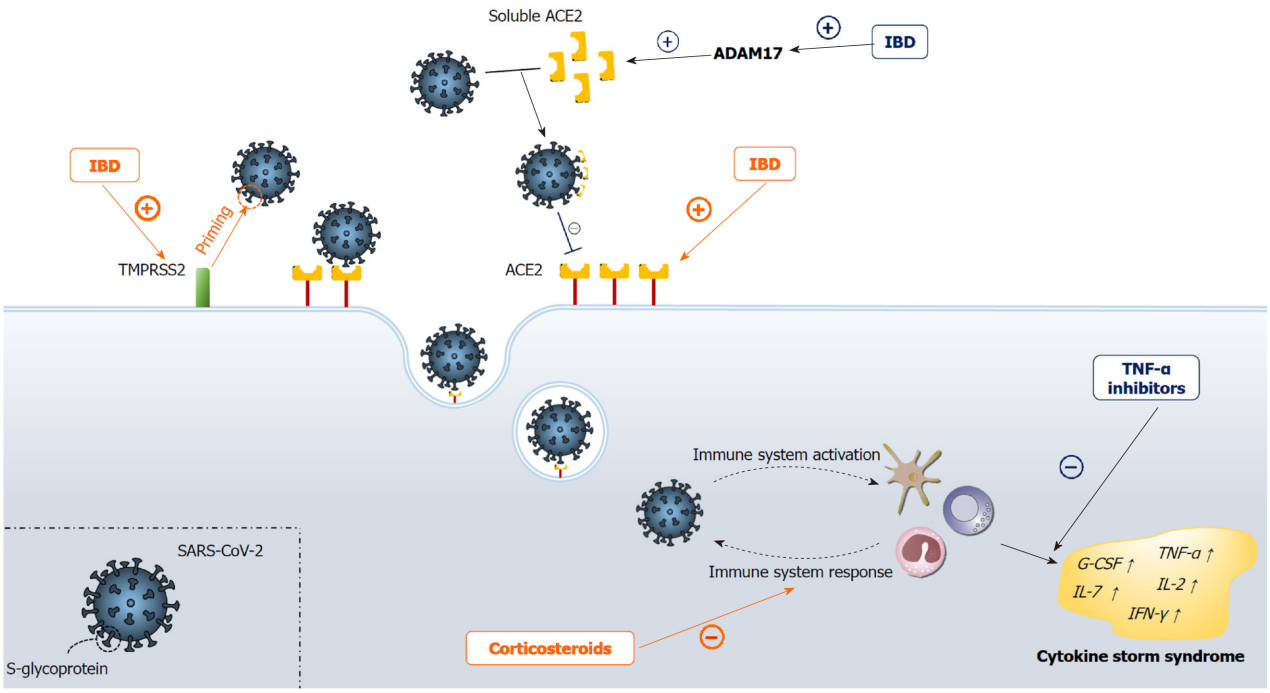


Figure 1 Pathogenetic pathway of intestinal severe acute respiratory syndrome coronavirus 2 infection with proposed inflammatory bowel disease effects. The blue lines represent inflammatory bowel disease (IBD)/IBD approach-mediated reduction in either coronavirus disease 2019 (COVID-19) acquisition or poor outcomes of it, whereas the red lines represent IBD/IBD approach-mediated increase in risk for COVID-19 acquisition or poor outcomes. IBD: Inflammatory bowel disease; ADAM17: A disintegrin and metalloproteinase 17; TNF-α: Tumor necrosis factor-alpha; ACE2: Angiotensin-converting enzyme 2; TMPRSS2: Transmembrane protease serine 2; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; IL: Interleukin; G-CSF: Granulocyte-colony stimulating factor; IFN-γ: Interferon-γ.

but, as discussed by Singh *et al*[36], the observed increase could be related to the fact that 5-ASA use may be a proxy for underlying UC in these circumstances. Namely, patients with UC have higher ACE2 levels (although, as we discussed, this is not a reliable indicator) and a population with UC tends to be older than a CD population [46], hence they are more prone to get tested, as Singh *et al*[36] argue. Despite the sensitivity of the fecal reverse transcription polymerase chain reaction (RT-PCR) test for the diagnosis of COVID-19, its diagnostic power still needs to be elucidated. D'Amico *et al*[47] hypothesized that fecal RT-PCR testing may be useful in IBD patients to distinguish disease re-exacerbation from SARS-CoV-2 superinfection, allowing better patient management and targeted therapy.

CLINICAL COURSE OF COVID-19 INFECTION IN PATIENTS WITH IBD

Since the detrimental effects of immunosuppressive agents on the host-cell defense against pathogens have been well-established, particular concern was raised with respect to the clinical course of COVID-19 in patients on various immunosuppressive therapies and ubiquitous therapeutic strategies for patients with IBD[15-17,48]. Furthermore, active IBD itself might worsen COVID-19 outcomes, as those patients are markedly frailer and more prone to adverse outcomes by virtually any infection[49, 50]. Finally, non-IBD patients with COVID-19 have high fecal calprotectin even after diarrhea resolves and COVID-19 patients with ongoing diarrhea have even higher levels in comparison with COVID-19 patients without diarrhea[51,52], suggesting that the presence of SARS-CoV-2 in the gastrointestinal tract is associated with greater intestinal inflammation. This indicates that COVID-19 could exacerbate inflammation and, subsequently, symptoms in IBD patients. However, it is very challenging to assign a symptom to the underlying disease, its exacerbation, or the concomitant infection, making these characteristics difficult to interpret. Of note, in the aforementioned studies[51,52], patients with diarrhea exhibited higher serum interleukin 6 concentrations, raising the possibility of more severe systemic inflammation in this group of patients[53].

Fortunately, an abundance of clinical studies demonstrated that most of the hypothesized adverse outcomes were not observed in IBD patients that acquired COVID-19. Nonetheless, in some cases this comorbidity seems to even dampen the deleterious effects of COVID-19. Considering the differences in the initial clinical presentation, although in concordance with the non-IBD population, fever and cough were the most common clinical findings. Further, COVID-19 positive IBD patients presented with diarrhea significantly more often than the general population[54-57]. This disparity could be associated with the influence of the underlying disease on the number of evacuations, justifying the greater percentage of diarrhea in IBD patients than in the general population. In contrast, the observed difference could also be due to the aforementioned exacerbation of IBD as a result of COVID-19 infection. The risk of severe COVID-19 outcomes, *i.e.*, the need for hospitalization, admission to the ICU, mechanical ventilation or death, were not higher among IBD patients in comparison to the general population, as demonstrated in multiple systematic reviews and meta-analyses[35,36,47]. These results are also in accordance with the latest data from the Surveillance Epidemiology of Coronavirus Under Research Exclusion for Inflammatory Bowel Disease (SECURE-IBD), an international web-based database where physicians are encouraged to report all cases of COVID-19 in patients with IBD[58]. A total of 4038 IBD patients with confirmed COVID-19 were reported in the SECURE-IBD database as of January 6, 2021 with 19% of patients in need of hospitalization, 3% admitted to the ICU, and 3% in need of mechanical ventilation, where the case fatality rate was 2%. Of note, in a study by Lukin *et al*[59], the authors included a control group consisting of non-IBD patients with COVID-19. Rather interestingly, death and ICU admission were numerically lower in the IBD group than in the control group. Although these results should be taken with caution, it is possible that certain IBD medications led to the blunting of the cytokine release syndrome and subsequently to more favorable outcomes.

The therapeutic choice emerged as a major determinant for COVID-19 prognosis in patients with IBD. Accumulating data implies that the use of systemic corticosteroids is associated with the highest risk of severe COVID-19 outcomes[35,36,47,58]. It is well known that corticosteroids affect the immune system *via* multiple mechanisms, including the inhibition of adhesion molecules, decreasing the expression of inflammatory cytokines and inducing apoptosis of activated lymphocytes. Moreover, in studies that tested the use of corticosteroids on MERS and SARS patients, authors have demonstrated a delayed viral clearance in patients receiving high-dose corticosteroids [60]. However, the effects of corticosteroid use on COVID-19 adverse outcomes in IBD is not as clear as it may seem. In a report from the large RECOVERY trial, in which the effects of dexamethasone on hospitalized COVID-19 patients were assessed, authors concluded that the use of dexamethasone resulted in lower 28-day mortality among those who were receiving either invasive mechanical ventilation or oxygen alone at the time of randomization but not among those receiving no respiratory support[61]. Furthermore, in a recently published meta-analysis by van Paassen *et al*[62], which included the largest number of studies and COVID-19 patients, the authors demonstrated the beneficial effects of corticosteroids use on short-term mortality and a reduction in the need for mechanical ventilation. Notably, the authors also found a signal of delayed viral clearance, but data in the studies were too uncertain to reach any firm conclusions. Three other meta-analyses that were conducted on this topic have rather conflicting reports. The WHO Rapid Evidence Appraisal for COVID-19 Therapies Working Group[63] concluded similarly to van Paassen *et al*[62], reporting that the administration of systemic corticosteroids was associated with lower 28-day all-cause mortality in comparison to placebo or usual care. Tlayjeh *et al*[64] found no difference in mortality or the necessity for mechanical ventilation, yet similarly to van Paassen *et al*[62], they observed a prolonged viral clearance time. Sarkar *et al*[65] demonstrated that in patients with COVID-19, corticosteroids may be associated with a twofold increase in mortality, yet their analysis was based on low-quality evidence with high variability. Considering the beneficial effects of corticosteroid use in COVID-19 patients, doubt was raised with respect to poor outcomes of their use in COVID-19 patients with concomitant IBD. It is possible that the corticosteroid use in these circumstances is merely an indicator from the subset of patients with active IBD who are predisposed to adverse outcomes. In fact, in a retrospective cohort study, Singh *et al*[66] reported that IBD patients who received corticosteroids up to 3 months before the diagnosis of COVID-19 had a higher risk of severe COVID-19 in comparison to patients who did not receive corticosteroids. Although the authors conducted an unadjusted analysis, because corticosteroid use in IBD is associated to worsening of the disease, these results could imply that in this setting corticosteroids were not the cause, but an indicator of higher risk for a severe COVID-19 clinical course[67].

Apart from corticosteroid use, as shown by multiple studies, the use of 5-ASA has been also associated with more severe COVID-19 outcomes[35,36,47,58,68]. This finding persisted even after controlling for confounding factors such as age, co-morbidities, IBD disease characteristics and corticosteroid use. Since mechanisms by which 5-ASA exerts its anti-inflammatory effect are rather disperse and include peroxisome proliferator-activated receptor-γ up-regulation, cyclooxygenase 2/prostaglandin E2 down-regulation, reactive oxygen species scavenging and many more[69], we could only hypothesize which of these pathways is responsible for more severe COVID-19 outcomes. Primarily, large, well-designed studies should be conducted to confirm that an increased risk really exists in the first place.

Immunomodulators, a group of medications used in IBD treatment that includes azathioprine, 6-mercaptopurine, and methotrexate have been known to inhibit the immune response to viral infections by multiple mechanisms[70,71]. However, the data regarding the role of immunomodulators in COVID-19 is quite reassuring, as conducted studies do not seem to demonstrate any difference in severe outcomes in comparison to the general population[35,36,47,58,68]. Since the effects of immunomodulators and corticosteroids on the suppression of the immune system are in part overlapping[72,73], we hypothesize that this provides further evidence toward the notion that the use of corticosteroids itself does not result in a more severe form of COVID-19. In fact, different results between the use of corticosteroids and immunomodulators from the above-noted studies could be due to the different clinical profiles of patients between the two groups, with corticosteroid use implying poor IBD control.

The most ambiguous results that emerged from observational studies is in relation to the use of biological agents, an immunosuppressive medication group used in the management of IBD which includes infliximab, adalimumab, golimumab, certolizumab pegol, ustekinumab and vedolizumab[74]. Biological agents, particularly TNF-α inhibitors, have been known to mitigate the host immune system response against infectious organisms, especially intracellular pathogens, such as mycobacterial, fungal and viral infections[75,76]. However, aside from the systematic review by Macaluso and Orlando[35] which showed no difference as opposed to the general population, available data suggests that patients treated with biological agents are significantly less prone to develop severe forms of COVID-19, distinctly in terms of mortality[36,58,68]. Accumulating evidence implies that COVID-19 severity is associated with a cytokine storm syndrome, an immune-mediated process characterized by hyperactivation of T cells and massive production of TNF-α, interleukin 2, interleukin 7, granulocyte-colony stimulating factor, interferon-γ (IFN-γ), IFN-γ–inducible protein 10, monocyte chemoattractant protein 1, and macrophage inflammatory protein 1-a [77]. Based on these findings, it is legitimate to hypothesize that the use of biological drugs which selectively inhibit specific cytokines or small molecules that simultaneously block multiple cellular pathways may play a role in the treatment of these patients. Interestingly, in two case reports, the remission of COVID-19 symptoms in IBD patients was achieved after treatment with biological agents[78,79]. This evidence is certainly insufficient to recommend the use of these drugs in COVID-19 management, yet they are reassuring for IBD patients, and shed light on biological agents as feasible therapeutic agents in COVID-19 treatment. In fact, there are several ongoing clinical trials that assess the efficacy and safety of these drugs in this setting (NCT-04344249 and NCT04425538).

Between the IBD subgroups, in all of the aforementioned reports[35,36,47,58,68], patients with UC had markedly worse outcomes than patients with CD. Singh *et al*[36] attribute this risk incrementation to the fact that patients with UC are more likely to be older and undergo different therapeutic modalities between the two groups[36,80]. However, since Singh *et al*[36] did not provide the age-adjusted comparison of outcomes, whether the poor outcomes of UC in contrast to CD are related to old age remains unclear. Regarding the difference in therapeutic modalities, patients with UC are more likely to be treated with 5-ASA, a treatment shown to pose a risk for more severe outcomes, whereas patients with CD are more likely to be treated with biological agents, treatment that seem to have a protective role in COVID-19 infection [81,82]. In addition, it is unclear if the observed disparity is due to the pathobiological differences between the two types of IBD, including the variability in expression of ACE2 and TMPRSS2.

THE IMPACT OF THE COVID-19 PANDEMIC ON IBD MANAGEMENT: PRESENT AND FUTURE PERSPECTIVES

Current guidelines for IBD treatment with respect to COVID-19

Two major organizations that cover the issues regarding IBD, the International Organization for the Study of Inflammatory Bowel Disease (IOIBD) and European Crohn's and Colitis Organization (ECCO) have partnered and provided a set of guidelines regarding the management of IBD in relation to COVID-19[83]. However, there are two important notions to accentuate regarding the present guidelines. Firstly, as currently there is no adequate evidence-based data, the recommendations are based on a consensus between a group of international IBD and infectious disease experts. Secondly, since the speed of publishing is slower than the amount of emerging data, organizations urged physicians to continue to check the IOIBD or the ECCO websites for the most up-to-date information.

The most important question that was raised in the guidelines concerns the IBD immunosuppressive therapies, *i.e.*, whether the infected patients should discontinue these therapies and if should, for how long. The current consensus is that recommencing these therapies should be influenced by the clinical severity of both IBD and COVID-19. Conceptually speaking, the greater the severity of IBD and the lesser the severity of COVID-19, the discontinuation of therapy should be shorter and vice versa. Experts suggest that for most patients, a symptom-based strategy is suitable [84]. According to this strategy, COVID-19 resolution is evaluated according to symptom onset (≥ 10 d) and clinical improvement. Current expert recommendations regarding immunosuppressive therapies are summarized in Table 1. Other aspects regarding recommendations, such as care for patients with IBD requiring hospitalization, priority for endoscopy, guidance for the infusion centers, management of pregnant IBD patients and a very practical set of ten "Do's" and "Don'ts" for IBD management during the COVID-19 outbreak were further discussed in the aforementioned IOIBD/ECCO guidelines[85-89]. Following the approval of several COVID-19 vaccines, the IOIBD experts have recently issued a statement regarding vaccination [90]. The expert group advised vaccinating all patients with IBD as soon as they are able to receive a vaccine, regardless of their immune-modifying therapies.

Impact of COVID-19 and lockdown on the management of IBD patients

There is a scarcity of data regarding the post-lockdown phase in terms of health-care procedures[91,92], let alone in the IBD population[93-96]. Early into the pandemic, hospitals were urged to restructure their daily activities to meet the needs of health care practitioners and to provide the facilities to treat COVID-19 patients. The restructuring of the health care system did not circumvent IBD management. Consequently, the risks of secondary harm emerged, as the latter resulted in reduced access to diagnostic endoscopy, lack of face-to-face clinics, difficulties in continuing day-case infusions, issues in performing routine blood and/or stool monitoring as well as patients' fears which may have reduced their attendance in hospitals[93]. Particular problems emerged in the pediatric population, since a delay in diagnosis and delayed treatment has the potential to result in serious repercussions, such as an impact on children's growth[97,98]. Recent Italian and Spanish surveys both demonstrated that the management of urgent activities and administration of biological therapies in both the lockdown and post-lockdown periods substantially maintained the pre-pandemic standards of care[93,94]. However, the surveys also highlighted that the reduction in number of visits, endoscopies and gastrointestinal ultrasounds observed in the lockdown but also in the post-lockdown phase could result in worse long-term outcomes[93,94]. A study in the pediatric population had similar conclusions regarding the quality of care[93]. This study accentuated concerns with respect to newly diagnosed IBD patients as this subgroup was diagnosed without a histological confirmation of the disease, which is a controversial exception that had to be adopted given the present special circumstances[93]. These patients were diagnosed *via* a combination of blood tests, radiological imaging, fecal calprotectin and exclusion of infectious causes, followed by multidisciplinary discussion. The commencement of the systemic immunosuppression in children without endoscopic or histological diagnosis was an additional concern, yet physicians could adapt in the beginning exclusive enteral nutrition as a first-line therapy as an induction strategy with multiple benefits [99]. Although a delay in exposing patients to systemic immunosuppression for 4-8 weeks is beneficial, after this time patients enter a period where immunomodulatory and biological therapy should commence, while simultaneously, the ability to conduct a full disease assessment beforehand may continue to be limited. Overall, experts

Table 1 Latest International Organization for the Study of Inflammatory Bowel Disease/European Crohn's and Colitis Organization recommendations for coronavirus disease 2019 infected inflammatory bowel disease-patients[101]

COVID-19 symptomatology	SARS-CoV-2 status	Recommendation
Asymptomatic	Non-tested	Do not withhold therapy (reduce corticosteroid use if possible)
Asymptomatic	Positive	Withhold therapy for 10 days[1]
Symptomatic	Positive	Withhold therapy until all of the following is fulfilled[1]: (1) At least 10 days has passed since symptoms onset; (2) Improvement in respiratory symptoms; and (3) Days without fever (without the use of antipyretics)

[1]If clinical presentations imply that therapy should not be postponed, despite meeting the clinical criteria stated above, guidelines recommend moving ahead with the scheduled treatment. COVID-19: Coronavirus disease 2019; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

agree that the implementation of a telemedicine approach has played an important role in maintaining the standards of quality of care in IBD management during the pandemic[93,94,100].

Future perspectives of IBD management

Even though COVID-19 had a range of detrimental effects on health care systems globally, it also opened a space for improvements in clinical practice, which could be used long after the COVID-19 pandemic resolves. One of those is the use of telemedicine, *i.e.*, the implementation of virtual technologies in routine clinical practice. An expansion in technological solutions and loosening restrictions on how telemedicine can be deployed and reimbursed have opened the way for telemedicine to become an integral part of clinical practice both now and in the future. In the IBD population, virtual appointments, multidisciplinary discussions, and improvement of networks by remote collaboration all provide the opportunity for better care within specific situations, with the simultaneous reduction of the transmission of infectious diseases. Today it is COVID-19, but in the future, it could be some other virus, especially as we are now more than ever aware of our susceptibility to a viral pandemic. IOIBD/ECCO issued a summary of the best strategies for IBD management *via* telemedicine, carefully covering every aspect of the patient-physician relationship [101]. Another very important aspect of telemedicine is its inexpensiveness. As a global economic crisis is imminent, this will actually become the critical reason for widespread implementation of telemedicine. Regarding the disease itself, although for now a lot of data substantiates the fact that COVID-19 does not influence the short-term prognosis for IBD patients, the long-term effects are quite unknown. We believe that poorer long-term outcomes will be mainly due to delayed diagnostic (especially endoscopy) therapeutic procedures and not COVID-19 itself. However, Gower-Rousseau *et al*[102] argue that most of the researchers too hastily concluded that the COVID-19 pandemic is relatively safe for IBD patients. They highlighted that the recently published, underpowered studies cannot provide answers for patients with IBD, or other infrequent diseases for that matter. Further, Gower-Rousseau *et al*. asserted that low-quality studies might even prompt misguided and harmful treatment decisions. On the contrary, they argue that well-grounded answers to these questions require complex epidemiologic risk and benefit analyses with an *a priori* sample size calculation and a removal of unwanted biases.

CONCLUSION

The initial fear of COVID-19 infection among patients in the IBD community that was based on available knowledge, now seems unnecessary. Accumulating data suggests that IBD is not a comorbidity that poses an increased risk for COVID-19 acquisition, except in patients treated with 5-ASA. Furthermore, although the outcomes of infected patients are largely dependent on the therapeutic modality by which they are treated, overall, IBD patients seem to have COVID-19 outcomes similar to the general population. This is in contrast to those on corticosteroids, as they currently seem to have a less favorable prognosis. Biological agents even dampen the detrimental effects of COVID-19 by inhibiting cytokine storm syndrome, according to the available data. However, preliminary data must be interpreted with caution, as the long-term effects

of both COVID-19 and IBD management during the COVID-19 outbreak are quite unknown. Finally, the COVID-19 outbreak could also change the future of IBD management, and management of the diseases in general, as telemedicine could dethrone face-to-face examinations in the following years.

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REVIEW

Management of hepatitis B virus infection in patients with inflammatory bowel disease under immunosuppressive treatment

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Abstract

Hepatitis B remains a significant global clinical problem, despite the implementation of safe and effective vaccination programs. The prevalence of hepatitis B virus (HBV) in patients with inflammatory bowel disease (IBD) largely follows the regional epidemiologic status. Serological screening with hepatitis B surface antigen (HBsAg), and antibodies to hepatitis B surface (anti-HBs) and core (anti-HBc) proteins is a key element in the management of IBD patients and, ideally, should be performed at IBD diagnosis. Stratification of individual cases should be done according to the serologic profile and the IBD-specific treatment, with particular emphasis in patients receiving immunosuppressive regimens. In patients who have not contracted HBV, vaccination is indicated to accomplish protective immunity. Vaccination in immunosuppressed patients, however, is a challenging issue and several strategies for primary and revaccination have been proposed. The risk of HBV reactivation in patients with IBD should be considered in both HBsAg-positive and HBsAg-negative/anti-HBc-positive patients, when immunosuppressive therapies are administered. HBV reactivation is preventable *via* the administration of prophylactic nucleot(s)ide analogues and should be the standard approach in HBsAg-positive patients. HBsAg-negative/anti-HBc-positive patients represent a non-homogeneous group and bear a significantly lower risk of HBV reactivation. Biochemical, serological and molecular monitoring is currently the recommended approach for anti-HBc patients. Acute HBV infection is rarely reported in IBD patients. In the present review, we outline the problems associated with HBV infection in patients with IBD and present updated evidence for their management.

Key Words: Hepatitis B virus; Inflammatory bowel disease; Reactivation; Immunosuppression; Vaccination; Prophylaxis

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Core Tip: The management of hepatitis B virus (HBV) infection poses significant challenges for patients with inflammatory bowel disease (IBD). Lower rates of vaccination for HBV have been reported in this population and immunization programs should be encouraged and intensively implemented. In addition, patients who receive immune-modifying therapies may develop suboptimal responses to vaccination. In the presence of present or past HBV infection, immunosuppressive therapies may increase the risk for reactivation of the virus with adverse clinical outcomes. Close surveillance and/or prophylactic anti-viral treatment may be employed depending on the status of HBV infection and the IBD-specific therapy.

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INTRODUCTION

Hepatitis B is among the most common infections worldwide and represents a major global health problem due to its potential for considerable morbidity and mortality. According to a World Health Organization report, in 2015, 257 million people were living with chronic hepatitis B infection[1]. In fact, it has been estimated that approximately one-third of the world's population has been exposed to hepatitis B virus (HBV). HBV infection is a frequent cause of both acute and chronic hepatitis. The latter may result in a variety of outcomes, which range from asymptomatic infection to end-stage liver disease with cirrhosis, hepatocellular cancer and death. Accordingly, it has been reported that HBV infection accounts for 5%-10% of liver transplantations[2]. On the other hand, nowadays, HBV infection is a preventable disease, due to the development and universal application of highly effective and safe vaccines against HBV.

Crohn's disease (CD) and ulcerative colitis (UC), collectively referred to as the inflammatory bowel diseases (IBDs), are immune-mediated diseases that manifest with chronic relapsing inflammation of the gastrointestinal tract. HBV infection may be of particular significance for patients with IBD, due to several factors. Firstly, although initially described as diseases of the West, since the second half of last century, IBDs have displayed accelerating incidence and prevalence rates in developing areas of the world[3-5]. As those regions include countries where HBV is highly prevalent, such trends translate to a constantly increasing number of patients with IBD that may be exposed to and infected with HBV. Secondly, there are reports indicating that patients with IBD may have increased rates of HBV positivity, while, at the same time, vaccination rates may be considerably low in this population. Finally, and more importantly, the mainstay of IBD treatment has been immunomodulatory drugs. Despite the shift from generalized immunosuppression with steroids or thiopurines to selective, targeted immunomodulation *via* biologics or small molecules, various degrees of immune response compromise are expected in IBD patients under treatment[6,7]. This state of acquired immunodeficiency may render patients with IBD more vulnerable to acquisition and to a more severe course of various infectious diseases, which include HBV infection. This also encompasses the danger for reactivating a previously latent HBV infection.

In this review, we will present the current status regarding the complex relationship between HBV and IBD, including updated epidemiological trends, screening and vaccination guidelines, and the associations between immunomodulatory treatment and the various clinical scenarios of anti-HBV immunity.

NATURAL HISTORY OF HBV INFECTION

HBV is a hepatotropic DNA virus that belongs to the Hepadnaviridae family. It is

transmitted sexually, parenterally (by contact with infected body fluids or blood) and perinatally, from a hepatitis B surface antigen (HBsAg)-positive mother to her child (vertical transmission). Following transmission, the HBV virion enters the hepatocyte using a cellular receptor, the sodium taurocholate co-transporting polypeptide (known as NTCP)[8]. The first step in HBV replication is the formation of covalently closed circular DNA (cccDNA) in the cell nucleus, a mini chromosome, which is a key structure for the longevity of the virus[9]. It is also known that fragments of HBV DNA integrate in the human DNA, but this integration is not vital for viral replication. After contact with the virus, the risk of chronic infection largely depends on the age of the subject, being 90% for infants, 25%-50% for toddlers, and 1%-5% for adults[10].

Chronic HBV infection consists of different phases with distinct serologic profiles, as have recently been described[11]. Phase I is the HBeAg-positive chronic HBV infection, formerly known as the immune tolerance phase, which is characterized by the presence of HBsAg and hepatitis B e antigen (HBeAg), very high HBV DNA levels and persistently normal transaminases, with no or minimal necroinflammation in the liver. The second phase is the HBeAg-positive chronic HBV hepatitis, which is characterized by high HBV DNA levels, increased transaminases and moderate to severe necroinflammatory activity and fibrosis in the liver tissue. This phase leads to seroconversion in the majority of cases, with loss of the HBeAg and appearance of antibodies to hepatitis B surface protein (anti-HBs). Phase III is the HBeAg-negative chronic HBV infection, previously known as the inactive carrier state, which is characterized by the presence of antibodies to hepatitis B e protein (anti-HBe), undetectable or low levels of HBV DNA, normal alanine aminotransferase (ALT) levels and HBsAg levels usually below 100 IU/mL. Phase IV is the HBeAg- chronic hepatitis, which is characterized by a moderate/severe necroinflammatory process in the liver, high HBV DNA levels and fluctuating liver enzymes. Phase V is the HBsAg-negative phase, also known as occult HBV infection, which is characterized by the absence of the HBsAg, positivity for antibodies to hepatitis B core protein (anti-HBc), with or without anti-HBs antibodies, usually normal ALT, and not necessarily measurable HBV DNA in the serum but detectable in the liver tissue.

The natural history of HBV depends on the phase of the infection; in patients with chronic hepatitis B, progression to cirrhosis is observed at an annual rate of 2%-2.5%, whereas HBeAg-negative patients exhibit a faster progression, at a rate of 8%-20% per year[12]. Thus, signs and symptoms of portal hypertension should be regularly sought for in this population, as should the degree of liver fibrosis *via* imaging. Furthermore, HBV is strongly associated with hepatocellular carcinoma and patients should be stratified according to the risk for malignancy *via* simple scores like the PAGE-B, which incorporates parameters like age, gender, and platelets[13]. Screening for hepatocellular carcinoma is based on abdominal ultrasounds at 6-mo intervals, with or without alpha-fetoprotein measurement.

HBV REACTIVATION

Reactivation of HBV is a distinct event in the natural history of the infection, that is signified by the recurrence of viral replication in patients with a quiescent disease status. According to the American Association for the Study of Liver Diseases[14], in HBsAg-positive patients, reactivation is defined as either at least 2 Log (or 100-fold) increase in HBV DNA compared with the baseline level or HBV DNA at least 3 Log (or 1000) IU/mL in a patient with previously undetectable HBV DNA or HBV DNA at least 4 Log (or 10000) IU/mL if the baseline level is not available. In HBsAg-negative, anti-HBc-positive patients, reactivation is considered as reappearance of the S antigen or the detection of HBV DNA. It is worth mentioning that there are patients who remain HBsAg-negative upon reactivation. This is genetically determined and related to the presence of additional N-linked glycosylation sites in the major hydrophilic region of the S antigen[15] and with the emergence of viral strains with mutated HBsAg[16]. HBV reactivation in patients under immunosuppression may have a deleterious effect. HBV reactivation presents with a wide range of manifestations, from asymptomatic, mild hepatitis to acute liver failure and death. In some cases, HBV reactivation precipitates the induction of chronic hepatitis B, potentially leading to liver cirrhosis and even hepatocellular carcinoma. Severe flares resulting in hepatic decompensation with an unfavorable outcome, commonly present with jaundice. A flare of hepatitis is defined as an ALT level above 100 U/L and at least 3 times higher than baseline. Patients with acute liver failure as a result of HBV reactivation present with higher HBV viral loads and lower IgM anti-HBc titers as compared with patients

with new onset HBV-related acute liver failure[17].

The major precipitating factor for HBV reactivation is the induction of an immunodeficient state in the host, more commonly *via* the administration of immunosuppressive therapies. This is due to the fact that HBV is controlled through the immunological system by specific T and B cells[18]. It follows that, in the presence of immunosuppression, the virus may regain its ability to proliferate and replicate[14]. This explains why HBV reactivation is of particular significance for patients with IBD, as the latter are often treated with therapies that modify the function of the immune system. It should be noted, however, that the large majority of the data originates from studies in oncology, hematology and rheumatology and that extrapolation of these data to IBD patients should be done with caution[19]. Nevertheless, immunosuppression does take place in treated patients and may be of relevance. As an example, tumor necrosis factor (TNF), which is the target of pivotal treatments for patients with IBD, is known to enhance virus clearance; hence, inhibition of TNF signaling enhances HBV replication[11]. Moreover, HBV has a glucocorticoid responsive element in its genome that is stimulated by the use of steroids, another frequently used therapy during flares of IBD[20].

Besides immunosuppression, HBV reactivation may also depend on both host and viral factors. Male sex has been associated with HBV reactivation in oncology patients [21]. HBsAg-positive patients carry higher risk for reactivation as compared to HBsAg-negative, anti-HBc-positive patients. Furthermore, higher HBV DNA levels before the start of immunosuppression confer an elevated risk of reactivation[22]. Finally, among HBsAg-negative patients, those who are anti-HBc-positive with detectable HBV DNA and undetectable anti-HBs before immunosuppression are more susceptible to reactivation[23].

After immunosuppression is imposed, HBV reactivation develops through sequential distinct stages[14,24]. At first, immunosuppression leads to an increase in HBV DNA, whilst the patient remains asymptomatic and transaminases are normal. Subsequently, hepatitis ensues with increased transaminases, with or without symptoms. In some cases, liver damage at this stage may lead to liver failure and even death. Once immunosuppression is either discontinued or reduced or/and antiviral treatment is initiated, a progressive decline of the HBV DNA is observed and the hepatitis flare resolves. In a small percentage of patients, despite such therapeutic measures, progressive deterioration of liver function may still be observed. Moreover, acute liver failure in patients under immunosuppression is associated with poor short-term prognosis and reduced 21-d overall survival compared with immunocompetent patients[25].

HBV PREVALENCE IN PATIENTS WITH IBD

HBV prevalence exhibits a geographic variability around the world, with areas of low (< 2%), medium (2%-7%) and high (> 8%) endemicity[11]. Highly endemic areas include Southeast Asia, the Pacific (excluding Japan and Australia), sub-Saharan Africa and some Eastern European countries. Areas with intermediate endemicity include South, Central and Southwest Asia, Israel, Japan, Eastern and Southern Europe, Russia and most of Central and South America. Low-endemic areas include North America, Western and Northern Europe, Australia, and parts of South America. The worldwide prevalence of HBV has changed over the last 30 years, due to immigration, improvement of the socioeconomic level and the implementation of mandatory vaccination.

The prevalence of hepatitis B in IBD patients has been investigated in several studies from Europe, Asia and the Americas (Table 1). Older studies from European countries showed higher incidence of hepatitis B core antibody (HBcAb) positivity among IBD patients[26,27]. Nevertheless, in more recent reports from Italy[28] and France[29], the prevalence of HBV infection among IBD patients was not different from that in the general population. Similarly, results of two Greek studies also reported prevalence of 2.3%-5% among IBD patients, which was in accordance to what was expected[30,31]. In addition, a study from Poland, a country with intermediate endemicity, also showed that the prevalence of HBV infection among IBD patients was comparable to that of the general population[32]. The most recent study by Losurdo *et al*[33], published in 2020, evaluated the burden of viral hepatitis in IBD and demonstrated that HCV was more frequent than HBV infection in IBD patients but with low overall prevalence.

Table 1 Studies investigating hepatitis B virus prevalence in inflammatory bowel disease patients

Ref.	Type of study; Country	Patients	HBsAg-positive	Anti-HBc-positive	Anti-HBs-positive	Comments
Losurdo *et al*[33], 2020	Single-center cohort; Italy	807 IBD	0.9%	7.7%		Similar to regional prevalence
		438 CD				
		369 UC				
Fousekis *et al*[31], 2019	Retrospective single-center; Greece	602 IBD	5.3%	13.4%	32.4%	Similar to regional prevalence
Silva *et al*[94], 2019	Cross-sectional; Brazil	306 IBD	0.7%			Similar to regional prevalence
		165 UC				
		141CD				
Chou *et al*[95], 2019	Retrospective; Taiwan	190 IBD	13.3%			Higher prevalence of HBsAg in IBD
		80 CD				
		110 UC				
Yeo *et al*[96], 2018	Prospective cohort; Korea	210 IBD	3.8%	26.2%		Treatment-naïve similar prevalence
		109 UC				
		101 CD				
Chen *et al*[35], 2017	Retrospective; China	980 IBD	7.9%	41.2%	46.6%	Higher prevalence of HBsAg in IBD
		334 UC	8.1%	52.7%	48.8%	
		646 CD	7.7%	35.3%	45.5%	
Harsh *et al*[34], 2017	Retrospective; India	908 IBD	2.4%			Similar to regional prevalence
		581 UC	2.2%			
		327CD	2.8%			
Waszczuk *et al* [32], 2016	Prospective cross-sectional; Poland	147 IBD		14.3%		Similar to regional prevalence
Chan *et al*[97], 2016	Retrospective cohort; China	406 IBD	5.7%			Similar to regional prevalence
He *et al*[98], 2015	Retrospective; China	449 CD	13.6%	25.4%	31.2%	Similar to regional prevalence
		226 UC	16.8%	30.1%	24.3%	
Ben Musa *et al* [47], 2014	Retrospective observational; United States	500 IBD	1.8%	3.2%		Similar to regional prevalence (screening rate 51%)
Huang *et al*[99], 2014	Retrospective; China	714 IBD	5.5%	40.6%	21.6%	Higher prevalence of HBsAg in IBD patients
Kim *et al*[37], 2014	Observational; Korea	513 IBD	3.7%			Similar to regional prevalence
		241 CD	4.1%			
		272 UC	3.3%			
Papa *et al*[28], 2013	Prospective; Italy	301 IBD	0.3%	7.3%		Similar to regional prevalence
Park *et al*[93], 2012	Retrospective; Korea	4153 IBD	4.1%			Similar to regional prevalence
		1521 CD	3.6%			
		1728 UC	4.6%			
Katsanos *et al*[30], 2010	Retrospective; Greece	482 IBD	2.3%			Similar to regional prevalence
Chevaux *et al* [100], 2010	Hospital-based; France	315 IBD			48.9%	Similar to regional prevalence
		252 CD	0.8%	2.8%		
		63 UC	1.6%	1.6%		
Loras *et al*[101],	Multicenter Hospital-based;	2076 IBD				Similar to regional prevalence

2009	Spain	1128 CD	0.6%	7.1%	17%	
		928 UC	0.8%	8%	14.9%	
		20 IC	0	5.3%	17.6%	
Tolentino *et al*[27], 2008	Hospital-based; Brazil	102 CD	0	43.3%		Higher prevalence of anti-HBc patients
		74 UC	2.3%	56.7%		
Esteve *et al*[102], 2004	Multicenter; Spain	80 CD	7.5%			Screening prior to anti-TNF treatment
Biancone *et al*[26], 2001	Multicenter; Italy	332 CD	2.1%	10.9%	14.4%	Higher prevalence of HBsAg in IBD
		162 UC	0.6%	11.5%	15.8%	

CD: Crohn's disease; HBc: Hepatitis B core protein; HBs: Hepatitis B surface protein; HBsAg: Hepatitis B surface antigen; IBD: Inflammatory bowel disease; TNF: Tumor necrosis factor; UC: Ulcerative colitis.

Studies from parts of the world with high HBV endemicity have also been published. In a report from India, the prevalence of HBV among 908 CD and UC patients was 2.8% and 2.2% respectively, both being comparable to the national prevalence of HBV[28]. Interestingly, HBV prevalence was higher in patients with intestinal tuberculosis than in IBD patients[34]. Data from China are contradictory; the prevalence of ongoing HBV infection in IBD patients paralleled that in the general population (7.86% and 7.3%)[29]. However, when assessing the prevalence of both present and past infection (HBsAg-positive and HBsAg-negative, anti-HBc positive) prevalence was significantly greater in IBD patients than in healthy individuals[35]. Additionally, in this group of patients, age above 30 years, UC and previous surgery were found to be the main risk factors[35]. Two studies from Korea, a highly endemic area for HBV with predominantly vertical/perinatal mode of transmission, demonstrated higher prevalence of HBV infection among IBD patients than in the western countries, but similar to the Korean general population[36,37]. It is noteworthy that, in the above studies, HBV DNA was not determined in anti-HBc-positive patients, and therefore the true frequency of occult hepatitis B was not determined.

Taken together, the majority of available evidence support the hypothesis that the cumulative prevalence of HBV in IBD patients parallels the national trends for HBV infection in each country. This is particularly true for European countries and, overall, indicates that IBD alone does not seem to constitute a risk factor for hepatitis B.

MANAGEMENT OF HBV INFECTION IN PATIENTS WITH IBD

All patients who have been diagnosed with IBD should be screened for their immunological status regarding exposure to HBV, preferably at the time of diagnosis. Unfortunately, surveys conducted by the European Liver Patients Association (commonly known as the ELPA) suggest that up to 90% of HBV-infected people in Europe are unaware of their condition[38]. It is, therefore, of great importance that patients be screened upon diagnosis and that the screening be performed in a pre-defined manner *via* the implementation of checklists provided by International societies[7,39,40]. It is obvious that such practice is of far greater significance for those patients who are scheduled to commence immunosuppressive therapy[7].

Initial screening should include testing for HBsAg, as well as anti-HBc and anti-HBs [7]. Based on the results from these tests, the immunization status may fall into one of three categories that should be then managed appropriately (Table 2).

HBsAg-negative, anti-HBs-negative, anti-HBc-negative patients

Patients who test negative for all three serological markers are susceptible to HBV infection upon contact with the virus. Currently, vaccination is recommended to all HBsAg-negative patients who are also negative for both anti-HBc and anti-HBs[7].

Encouraging immunization is very important. Treating gastroenterologists should explain the advantages of vaccination, while reassuring patients and providing them with vaccine-related information, as this will lead to better compliance and increased participation in vaccination programs[41,42]. Constant training of the physicians and participation in educational activities is similarly important, because gaps in the knowledge of gastroenterologists regarding vaccinations have been reported[43].

Table 2 Serologic profiles after initial screening

	HBsAg	Anti-HBc	Anti-HBs
Susceptible	Negative	Negative	Negative
Immune due to vaccination	Negative	Negative	Positive
HBV infection	Positive	Positive	Negative
Resolved HBV infection or occult HBV infection	Negative	Positive	Positive or negative

HBc: Hepatitis B core protein; HBs: Hepatitis B surface protein; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus.

Implementation of a thorough guidance regarding vaccines has been shown to improve the overall adherence to vaccination guidelines[44].

Overall, vaccination rates in IBD patients have been reported to be low[45]. Memled *et al*[46] reported an overall vaccination rate of 28%, which reflects the fact that immunization history is often omitted. Even in tertiary centers, only half of IBD patients may have been screened for HBV[47]. Nevertheless, vaccination attitudes may be changing, nowadays, as improvements in HBV immunization practices for IBD patients have been documented during the last decades. According to Shah *et al*[48], only 8.1% of patients were vaccinated in 2003, *vs* 43.2% in 2011[48]. In this study, between 2003-2011, an overall HBV screening rate of 23.7% in a population of IBD patients under anti-TNF treatment was recorded. Another study from the Netherlands, spanning from 2000 to 2010, showed that the screening rates increased from 36% to 49% during the last 2 years of the study[49]. According to a more recent study of 1834 anti-TNF-naïve patients, HBV screening rates significantly improved between 2010 and 2019 (64% and 87.4% respectively)[50].

Available vaccines for HBV constitute the first- (plasma-derived), second- (yeast- or mammalian-derived recombinant major S antigen) and third-generation (major S and pre-S1 and -S2 proteins) vaccines (Table 3). Vaccines are further differentiated into single antigen vaccines, such as Engerix-B® (GlaxoSmithKline Biologicals, Rixensart, Belgium), HBVaxPRO® (Sanofi-Pasteur, Lyon, France), Recombivax® (Merck & Co, Inc, Kenilworth, NJ, United States), Heplisav-B® (HepB-CpG; Dynavax Technologies, Emeryville, CA, United States), Fendrix® (GlaxoSmithKline Biologicals), Sci-B-Vac® (VBI Vaccines, Cambridge, MA, United States) and combination vaccines like Twinrix® (GlaxoSmithKline Biologicals) (HBV + hepatitis A virus) and Pediarix® (GlaxoSmithKline Biologicals) (HBV + diphtheria/tetanus/whooping cough (pertussis) + polio).

The standard vaccination schedule in IBD patients is the same as that with the general population, which consists of three standard doses of rHBAg (20 μg) at months 0, 1 and 6, although deviations do occur in practice. For example, Indian guidelines recommend a double-dose, accelerated three-dosing scheme of 40 IU/mL in 0-1-2 mo[51]. Furthermore, in 2018, a novel recombinant vaccine was approved (Heplisav-B® - HepB-CpG), which bears a unique adjuvant sequence and is administered in two doses, 1 mo apart. This two-dose vaccine has shown promising results in individuals with risk factors for hyporesponsiveness (see below) but has not yet been tested in IBD patients specifically[52].

After completion of the dosing schedule, titers of anti-HBs should be checked within 1-3 mo to confirm the establishment of adequate anti-HBV immunological status, which is signified by detection of anti-HBs antibodies in the serum. In healthy individuals, HBV vaccination confers > 90% protective immunity. In contrast, it has been demonstrated that response rates after HBV vaccination are reduced compared with healthy individuals[53]. Indeed, it was shown that patients with future diagnosis of IBD had suboptimal vaccination response even before the clinical manifestation of IBD[54]. Based on a meta-analysis by Jiang *et al*[53], which included 13 studies with 1688 patients, the pooled response rate to vaccination for HBV among IBD patients was 61%. Rates were not affected by the specific diagnosis of CD or UC[55]. Not only are the rates of anti-HBs positivity decreased in patients with IBD but the titers of antibodies are also lower. In various studies, the reported average anti-HBs titers in healthy individuals after successful immunization were 720[56] and 822[57] IU/mL. In comparison, IBD patients under anti-TNF therapy had reported average values around 245 IU/mL[56]. This was also confirmed by another study by Belle *et al*[58], wherein median anti-HBs levels after vaccination were significantly lower in immunosuppressed patients (253 IU/mL *vs* 497 IU/mL). The significance of such differences is further exemplified by the fact that post-vaccination targets for anti-HBs titers may

Table 3 Available vaccines

	Company	Antigen - Adjuvant	Posology	Dosing
Engerix-B®	GlaxoSmithKline	Rec. major S Ag (yeast) Aluminum	1 mL	3- or 4-dose
			20 mcg	0-1-6 mo
				0-7 d-21 d-12 mo
HBVaxPRO®	Sanofi-Pasteur MSD	Rec. major S Ag (yeast) Aluminum	1 mL	3- or 4-dose
			5 mcg	0-1-6 mo
			10 mcg	0-1-2-12 mo
			40 mcg	
Fendrix®	GlaxoSmithKline	Rec. major S Ag (yeast) Aluminum + 3-O-desacyl-4 - monophosphoryl lipid A	0.5 mL	4-dose
			20 mcg	0-1-2-6 mo
Heplisav-B®	Dynavax	Rec. major S Ag (yeast) HepB-CpG ligand	0.5 mL	2-dose
			20 mcg	0-1 mo
Twinrix®	GlaxoSmithKline	In.HAV + Rec. major S Ag (yeast) Aluminum	1 mL	3-dose
			720/20 mcg	0-7-21 d
				0-1-6 mo
Sci-B-Vac®	VBI Vaccines	Major S Ag, minor pre-S1 + pre-S2 Ag (mammalian cell) Aluminum	1 mL	3-dose
			10 mcg	0-1-6 mo
Pediarix®	GlaxoSmithKline	DTaP + inactivated poliovirus + Rec.S (yeast) Aluminum	0.5 Ml	3-dose
			10 mcg	(6-8 wk interval)

Ag: Antigen; DTaP: Diphtheria-tetanus-whooping cough (pertussis); In.HAV: Inactivated hepatitis A virus; Rec.: Recombinant.

differ between IBD patients and healthy controls. In immunocompetent individuals, a titer above 10 IU/mL is considered adequate; whereas, in immunosuppressed patients, a higher titer, of 100 IU/mL, is considered protective[51]. Indeed, according to Loras *et al*[59], a distinction is made based on the quantity of the antibodies in the serum between seroprotection for titers > 10 IU/mL and effective vaccination for titers > 100 IU/mL.

Factors that have been associated with an inadequate immune response to HBV vaccine have included older age, immunosuppressive therapy, and incomplete dosing (< 3 doses administered)[53,60]. In addition, Altunoz *et al*[55] noted a negative correlation between disease activity and adequate antibody response both for CD and UC. In another study, it was found that ileal disease correlated with lower responses to Engerix-B®. From an interesting perspective, gut microbiota was recently implicated as a regulator of immune response to vaccination. Experimental data from germ-free mice showed reduced response rates after vaccination, which were ameliorated after establishing normal microbiome[61]. As patients with IBD demonstrate intestinal dysbiosis, it could be hypothesized that the latter may negatively affect immunization in a similar manner. Nonetheless, such a concept has yet to be proven.

The major factor, however, that has been studied in relation to the efficacy of HBV vaccination in IBD patients has been the administration of immunomodulatory therapies[53]; hence, this has been the subject of research and discussion, given the fact that it affects the majority of people with CD or UC. Therefore, it is generally recommended to proceed with HBV vaccination ideally at the time of IBD diagnosis and preferably before initiating treatment. Among all immunosuppressive therapies anti-TNF treatment is a major negative influencer of HBV vaccination, as stated in the meta-analysis of Jiang *et al*[53]. The effect of anti-TNF treatment on vaccine efficacy in patients with IBD was assessed by Gisbert *et al*[62], who reported a response rate of 46%. On the other hand, in a European study, the response rates after primary vaccination with Engerix-B® in IBD patients, despite being lower in comparison with healthy individuals, were not adversely associated with the use of biologics and immunomodulators[58]. In the study of Loras *et al*[59], total response rates of 59% after first and second vaccination attempts were observed in patients treated with anti-TNF

monotherapy; whereas, in patients under combination therapy, only a 38% seroprotection rate was observed, highlighting the negative effect of combinatorial immunosuppression. Among the various anti-TNF biologics, the use of infliximab correlated with lower antibody response rate (16.7%) compared with adalimumab (48.4%)[56]. In the same study, ustekinumab exhibited 72% antibody response after vaccination. Treatment with vedolizumab does not seem to influence immune response after vaccination based on anti-HBs titers that were determined in the context of a randomized double-blind placebo control trial[63]. These findings are confirmed by recent data from Harrington *et al*[64], whereby 62.5% of IBD patients treated with vedolizumab achieved an anti-HBs level above 10 mIU/mL after a three-dose standard vaccination scheme with Engerix-B®, which is comparable to the response of immunocompetent patients. Data regarding the effect of treatment with tofacitinib on the vaccination efficacy in IBD patients are scarce. Studies in rheumatology show that it is associated with diminished response to pneumococcal but not influenza vaccination[65]. In a small retrospective study of patients with rheumatoid arthritis on tofacitinib, only 2 HBsAg-positive patients, who did not receive prophylaxis, developed reactivation[66].

Based on the aforementioned results, it appears that patients with IBD who are treated with immune-modifying agents may exert suboptimal immunization status after HBV vaccination. Consequently, strategies to improve efficacy have been implemented with various results (see next paragraph). Such approaches include accelerated, repeated or increased dosing, whereas different vaccines may also display diverse efficacies. Nevertheless, the most valuable approach may be the selection of the appropriate timepoint for vaccination. In fact, early vaccination of young patients who have not received any immunosuppressants is the best strategy for optimal immune response.

Gisbert *et al*[67] tested a protocol combining both increased dose and shorter intervals of vaccination using double-dose of Engerix-B® given in 0-1-2 mo. This accelerated scheme achieved an improvement in immunization rates from 41% to 75% compared with the standard protocol. Loras *et al*[59] used the same scheme for the vaccination of IBD patients under anti-TNF treatment and found a 57% response rate. A study by Chaparro *et al*[68] compared the double-dose Engerix-B® vaccine given in a four-dose schedule (0-1-2-6 mo intervals) with the single-dose Fendrix® given in the same intervals. The two vaccines demonstrated equivalent effectiveness rates but the four-dose protocol exhibited enhanced efficacy (range: 68%-75%) in achieving anti-HBs titers above 100 IU/mL. In another recent study by Haykir *et al*[56], comprising a standard schedule high-dose immunization program for a mixed population of IBD and rheumatology patients under immunosuppression, did not significantly improve the immune response. Similarly, use of third-generation vaccines (*e.g.*, Sci-B-Vac®) did not show any additional benefit compared with second-generation vaccines (*e.g.*, Engerix-B®)[69], despite elucidating higher response rates in immunocompetent healthy individuals. Taken together, these studies show that intensified protocols may accomplish higher rates of effective immunization. The variety of such approaches indicates that each IBD center should test and propose its own protocols, based on local experience, efficacy rates and available options.

Patients who have been effectively immunized should be monitored at least every 2 years, by assessing anti-HBs titers. It has been shown that 18% of patients lose their antibodies on a yearly basis[69,70]. In the case that the first immunization attempt is unsuccessful, several revaccination strategies have been proposed (Table 4). The most common approach in this setting entails the repetition of a standard three-dose scheme. Indeed, a three-dose revaccination schedule yielded better response rates (62.9%) than a single-dose or 2 additional doses (40.2%), when evaluated according to patient ability to mount an anti-HBs titer above 10 IU/mL, emphasizing the need of completion of the three-dose schedule[71]. The same three-dose revaccination approach in the study of Cossio-Gil *et al*[60] achieved a response rate of 52.8%. However, in these studies, an anti-HBs threshold of 10 IU/mL, instead of 100 IU/mL, was used. Levels of anti-HBs between 10-100 IU/mL after first vaccination are correlated with effective protective immunity characterized by obtaining anti-HBs > 100 IU/L after revaccination[59]. The response rate for patients older than 35 years who initially developed anti-HBs titer < 10 IU/mL was only 25% after repeat vaccination[59].

The Turkish society of gastroenterology recommends a response-guided approach based on anti-HBs titers after primary vaccination[72]. In patients with undetectable and/or < 10 IU/mL anti-HBs levels a double-dose, 0-1-6 mo complete revaccination scheme is proposed; whereas, in patients with anti-HBs levels between 10-100 IU/mL, a single double-dose booster dose is administered[72]. In healthy individuals without

Table 4 Revaccination studies for hepatitis B virus in inflammatory bowel disease

Ref.	Study	Patients, *n*	Strategies	Response rate for anti-HBs
Pratt *et al*[71], 2019	Retrospective cohort	149	3-dose schedule *vs* 1 or 2 doses	62.9%
				40.2% (> 10)
Cossio-Gil *et al*[60], 2015	Retrospective cohort	53	3-dose schedule	52.8%
Loras *et al*[59], 2014	Prospective	389	Double-dose 0-1-2	31.3% (> 100)
				44.4% (10-100)

HBs: Hepatitis B surface protein.

adequate immune response after primary vaccination with Engerix-B®, the use of a different vaccine (*i.e.*, Fendrix® or HBVaxPRO®) for revaccination resulted in improved response rates[73]. However, this practice has not been evaluated in IBD patients. At present, the optimal revaccination protocol in this population is unknown and further studies are needed. Vaccination for hepatitis B is generally considered safe. Injection site reactions and mild systemic adverse events are the most commonly reported problems[1]. HBV vaccination does not influence the course of IBD.

HBsAg-positive patients

Patients who test positive for HBsAg have ongoing HBV infection and are at the highest risk for reactivation if their disease is quiescent at baseline. Those patients should be initially tested with complete blood count and for levels of ALT, aspartate aminotransferase, albumin, and HBV DNA, as well as undergoing liver stiffness measurement and tests for other hepatitis virus (*e.g.*, hepatitis D virus). Results from those tests will help assess HBV status and evaluate the need for antiviral treatment and monitoring[7,11]. Patients who have HBsAg-positive and HBeAg-positive or -negative chronic hepatitis B [HBV DNA > 2000 IU/mL, ALT > upper limit of normal and/or at least moderate liver necroinflammation or fibrosis] need to receive antiviral treatment, irrespective of type of immunosuppression[11]. HBsAg-positive patients with IBD who will commence treatment with immune-modifying medications are at risk for reactivation of HBV. Factors that relate to HBV reactivation in IBD patients include infection status, level of immunosuppression and duration of immunosuppressive therapy.

Various guidelines have been offered as to the optimal course of action regarding the management of HBsAg-positive patients with IBD[7,11]. As a general rule, all HBsAg-positive patients with either chronic infection or hepatitis should receive prophylactic antiviral treatment before starting any type of immunosuppressive treatment[7,11,51]. The only exception is the statement from the American Gastroenterological Association (AGA)[74], whereby guidelines are diversified according to the estimated risk for HBV reactivation into high (> 10%), moderate (1%-10%) and low (< 1%) risk. In IBD patients, the therapy conferring high risk of reactivation is prednisone ≥ 10 mg daily for ≥ 4 wk. Therapies with moderate risk include TNF-α inhibitors, cytokines or integrin inhibitors and prednisone < 10 mg daily for ≥ 4 wk. Low risk for reactivation is conferred by treatment with traditional immunosuppressive agents (azathioprine, methotrexate) and any dose of oral steroids for ≤ 1 wk or low dose (< 10 mg daily) for ≥ 4 wk. Accordingly, high and moderate risk individuals should receive prophylactic nucleos(t)ide therapy, whereas patients at moderate risk are given the option for monitoring. The AGA recommends that antiviral treatment should be continued for at least 6 mo after discontinuation of the immunosuppressants[74]. In patients at low risk for reactivation, monitoring is sufficient. In addition, combination of immunomodulatory medications increases the risk of reactivation. In a multicenter Spanish study, Loras *et al*[59] demonstrated a pronounced risk of HBV reactivation in HBsAg-positive patients receiving ≥ 2 immunosuppressants without antiviral prophylaxis.

It should be noted that these recommendations are primarily based on data derived from oncology, rheumatology and hematology studies[75-77]. The extension of these conclusions in IBD patients is precarious, since differences do exist. In particular, the duration of treatment differs considerably between the two groups. Chemotherapy is usually offered for a finite number of treatment cycles. In contrast, IBD treatments may last life-long. In relevance to this, it should be remembered that HBV is not a direct

cytopathogenic virus and that liver cell damage in chronic hepatitis B is the result of immune system activation against the infected cells. As a result, a hepatitis flare is not observed during the time of maximal immunosuppression but at a later time point. Thus, anti-viral therapy should be continued for at least 12 mo after the cessation of any immunosuppressive therapy in patients with immune-mediated diseases, including IBD[78].

Nucleos(t)ide analogs are the preferred antiviral therapy in patients with IBD who will receive immunosuppression, as long-term treatment is an effective and safe strategy. In contrast, the use of pegylated-interferon is discouraged. The third-generation antivirals of tenofovir, entecavir and tenofovir alafenamide are recommended, due to their high antiviral activity with practically no resistance (antivirals with high resistance barrier). Entecavir or tenofovir alafenamide should be preferred over tenofovir in patients above 60 years of age, in patients with a history of bone disease (*i.e.*, fracture, osteoporosis, chronic steroid use), and in patients with renal dysfunction (with estimated glomerular filtration rate < 60 mL/min/1.1, albuminuria > 30 mg/24 h, or low phosphate < 2.5 mg/dL)[11]. Lamivudine is a good antiviral for short-term use, as the rate of 1-year and 2-year resistance is 20% and 30%, respectively. Given that immunosuppressives for IBD patients are long-term therapies, the use of lamivudine is discouraged. In fact, in a small published study, it was shown that 6 out of 8 IBD patients who received lamivudine prophylaxis required a change to newer antivirals[30].

Antiviral treatment should ideally start 2 wk prior to the commencement of immunosuppression and should be continued for at least 12 mo after treatment cessation, provided that the underlying HBV infection is quiescent.

According to a series of patients with chronic HBV infection and IBD with a long-term follow up (20 years), the natural history of HBV is not affected in this group of patients[36]. Nevertheless, an association between chronic viral hepatitis and non-alcoholic fatty liver disease has been shown in patients with IBD. In a recent study by Losurdo *et al*[33], IBD patients with concurrent chronic viral hepatitis present more frequently with diabetes, wide waist circumference and increased liver stiffness. In addition, the frequent use of steroids, is further considered a risk factor for NAFLD and the combination of liver steatosis and viral hepatitis may sensitize the liver and render it more vulnerable to developing liver-associated complications. These facts further emphasize the need for prophylaxis and treatment strategies for HBV in patients with IBD.

HBsAg-negative/anti-HBc-positive patients

Patients with isolated anti-HBc antibodies on serological testing represent a non-homogenous population. This serologic profile reflects either a false positive result particularly in regions of low endemicity or corresponds to the 'window' period before the appearance of anti-HBs, as in the case of resolved HBV infection. Moreover, isolated anti-HBc antibodies in the presence of undetectable anti-HBs may be found in a patient with resolved HBV infection, due to waning immunity after many years or to treatment with immunomodulating therapy. The latter scenario is of potential significance for the IBD patient, as it carries risk for HBV reactivation. This risk is lower than in HBsAg-positive patients, and is estimated to be between 4%-5%, based primarily on findings from oncological studies. In a meta-analysis by Cholongitas *et al*[79], the risk of HBV reactivation in anti-HBc-positive patients with non-hematological diseases was 3.6%. Quantification of anti-HBc antibodies can help distinguish occult hepatitis B infection from a past HBV infection, with a cutoff of 6.6 IU/mL[80]. Detection of anti-HBc antibodies serves as a surrogate marker of occult HBV infection, which is defined as the detection of HBV DNA in the liver tissue (gold standard) or in the blood[23].

The prevalence of occult HBV infection in this particular subgroup of patients varies, depending on the HBV DNA threshold used (200 U/mL) and ranges between 11%-89%[81]. More recent data indicate detectable HBV DNA in 0-27% of patients with exclusive anti-Hbc positivity[82]. When IBD populations were exclusively examined, the rates of anti-HBc positivity were highly dependent on the HBV endemicity. Data from low endemicity areas such as Italy and France range from 7.7% [33] to 0.6%[29] respectively, whereas data from eastern Europe (intermediate endemicity region) indicate a prevalence of 12%. In an HBV endemic area, anti-HBc positivity was 41.2%[35]. The risk of HBV reactivation depends also on the specific state of immunodeficiency, including the particular immunosuppressive therapy, being significantly higher in patients with malignant hematological diseases and especially with the use of the anti-CD20 agent rituximab.

The first case of HBV reactivation in an IBD patient with isolated anti-HBc positivity was reported in 2006, involving a woman with CD who was treated with infliximab [83]. Ever since, studies assessing the reactivation probability in patients under anti-TNF treatment have been performed, ultimately confirming a low risk of reactivation. Indeed, the reported reactivation rates vary between 3.13%[84] and 7%[59]. It has been reported that the risk of reactivation may be higher with the use of infliximab than with the other anti-TNFs[85].

When an anti-HBc-positive patient is scheduled to start therapy with immune-modifying medications, he should have an evaluation for HBV DNA presence in the blood. In the case of detectable HBV DNA, the patient is treated as being HBsAg-positive. The management of patients showing negativity for HBV DNA is not equally straightforward, particularly in patients with IBD, as literature is scarce. According to the AGA guidelines, anti–HBc-positive, HBV DNA-negative IBD patients who receive corticosteroids in high dose (> 20 mg prednisone daily) or moderate dose (10-20 mg prednisone daily for ≥ 4 wk) and/or anti-TNFs should receive antiviral prophylaxis over monitoring[74]. Antivirals should be continued for at least 6 mo after stopping immunosuppressants. It should be noted, however, that the level of evidence is "weak recommendation/moderate quality evidence" and, thus, the alternative for monitoring is also an acceptable option. According, to the European Association for the Study of the Liver (commonly referred to as the EASL) and the European Crohn's and Colitis Organisation (commonly referred to as the ECCO) guidelines, surveillance is strongly indicated and includes monitoring of transaminases, HBsAg, anti-HBs antibodies and HBV DNA, in the first month of treatment and every 3 mo thereafter. Pre-emptive antiviral therapy is commenced in the case of HBsAg seroconversion or HBV DNA detection. If monitoring cannot be ensured, it would be prudent to adopt the approach of the AGA and start prophylactic antiviral therapy. Accessibility to regular testing and follow-up as well as patient preferences should also be incorporated in decision-making. Treatment with azathioprine, 6-mercaptopurine or methotrexate in anti-HBc-positive patients carries a low risk of reactivation.

The frequency of anti-HBs in anti-HBc patients is approximately 75%. Emerging evidence suggests that HBV reactivation is most likely in those with low or undetectable anti-HBs levels[86]. The protective role of anti-HBs was illustrated in a meta-analysis by Paul *et al*[86], where patients with detectable anti-HBs had 79% lower odds of reactivation. Nevertheless, in the same meta-analysis, it is noted that HBV reactivation still occurs in patients with both anti-HBc positivity and anti-HBs positivity. In lymphoma patients, undetectable anti-HBs or titers of 10-100 mIU/mL at baseline are independent predictive factors for reactivation compared with anti-HBs titers of ≥ 100 mIU/mL[87]. Furthermore, in lymphoma patients treated with chemotherapy, quantification of anti-HBc and anti-HBs might help to predict HBV reactivation. High anti-HBc (6.41 IU/mL) and low anti-HBs (< 56.48 mIU/mL) at baseline are associated with a higher risk of reactivation (hazard ratio: 517.29; $P < 0.001$)[88]. However, this approach has not been evaluated in IBD patients. The proposed strategy according to ECCO guidelines for patients with anti-HBc and/or anti-HBs positivity is monitoring[7].

Acute HBV infection

Acute HBV infection during the course of IBD is a rare occurrence. Limited data exist in the literature and only few clinical cases have been described. Data from these case reports are inconsistent, underlining the variable evolution of acute HBV infection in immunocompromised IBD patients. In the case of a patient who developed acute hepatitis B, infliximab was temporarily discontinued, until antiviral treatment was commenced without further complications[48]. In contrast, a young CD patient on infliximab developed acute HBV infection that rapidly evolved to fatal liver failure [89].

In healthy adult individuals, acute HBV infection leads to resolution in approximately 90% of cases; whereas, in immunosuppressed patients, spontaneous recovery rates are reduced[90]. The diagnosis of acute hepatitis B infection is based on the detection of IgM anti-HBc. Yet, anti-HBc IgM are also detected during exacerbations of chronic hepatitis B. Quantitative measurement of anti-HBc IgM may be helpful in differentiating acute HBV infection from a flare of a chronic infection, as lower levels are detected in the latter[91]. Specific threshold values depend on the assay used. For instance, when applying chemiluminescent immunoassay (also commonly known as CLIA), levels > 10 show 100% sensitivity and 99% specificity[92] and levels > 8 show sensitivity of 96.2% and specificity of 89.7%[93]. Similarly, for HBV DNA, lower levels are observed in acute *vs* a flare of chronic HBV infection[91].

Kinetics of HBsAg have been used to predict the likelihood of chronicity, as a 50% reduction within 4 wk is predictive of resolution of HBV infection[91]. Prevention of liver failure is the therapeutic goal in acute hepatitis B in immunocompetent adults. This is why only patients with a protracted course or coagulopathy are candidates for antiviral treatment. In immunosuppressed patients, however, timely initiation of nucleos(t)ide analogues is suggested in order to avert the progression to liver failure.

CONCLUSION

Despite the implementation of mandatory vaccination of newborns against HBV, hepatitis B remains a significant global clinical problem. As the prevalence of IBD is also growing around the world, a substantial number of CD and UC patients with concomitant HBV infection remains and new cases are expected to occur. Screening of IBD patients with HBV serology is of paramount significance and should be actively communicated among patients and ideally implemented at diagnosis on IBD. The initial vaccination approach for IBD patients is largely similar to the recommendations for the general population, despite a lower reported vaccination efficacy, with considerable variation in revaccination strategies. It should be kept in mind that immunosuppressive treatment increases the risk of HBV reactivation; thus, close monitoring of patients at-risk is required. In the case of HBsAg positivity, antiviral prophylaxis is offered to IBD patients regardless of type of immunosuppression. On the other hand, HBsAg-negative, anti-HBc-positive patients represent a low reactivation risk group, for whom monitoring according to international and local guidelines is indicated.

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MINIREVIEWS

Worldwide management of hepatocellular carcinoma during the COVID-19 pandemic

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Abstract

The coronavirus disease 2019 (COVID-19) pandemic has impacted hospital organization, with the necessity to quickly react to face the pandemic. The management of the oncological patient has been modified by necessity due to different allocation of nurses and doctors, requiring new strategies to guarantee the correct assistance to the patients. Hepatocellular carcinoma, considered as one of the most aggressive types of liver cancer, has also required a different management during this period in order to optimize the management of patients at risk for and with this cancer. The aim of this document is to review recommendations on hepatocellular carcinoma surveillance and management, including surgery, liver transplantation, interventional radiology, oncology, and radiotherapy. Publications and guidelines from the main scientific societies worldwide regarding the management of hepatocellular carcinoma during the COVID-19 pandemic were reviewed.

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Core Tip: Hepatocellular carcinoma is one of the most common cancers in the world. The aim of this review is to focus on the impact of the coronavirus disease 2019 pandemic on the management of patients with hepatocellular carcinoma and to verify how multidisciplinary management has changed to face the necessity of hospital reorganization.

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INTRODUCTION

In December 2019, the world experienced a new coronavirus from China, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is responsible for a respiratory disease with the characteristics of a worsening interstitial pneumonia, later called coronavirus disease 2019 (COVID-19). This virus spread quickly across all continents generating a global pandemic, officially declared on March 11, 2020, which has put hospitals around the world under pressure as they were unprepared to face a very high number of hospitalizations in intensive care units and medical wards[1]. This affected not only intensive care, but clinical services of cancer patients as well. In fact, the recruitment of a large part of medical staff in the aforementioned departments in order to contain and fight this new health emergency caused serious problems in the management of all other wards with consequent delays in the care and surveillance of patients with other pathologies and, in particular, of those with oncological disease[2,3]. Moreover, cancer patients are at major risk to contract COVID-19 infection both from nosocomial exposure and due to their immunocompromised states [4].

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world, which accounts for approximately 6% of all cancer incidences[5] and is nowadays considered a global health problem[6]. Compared to other neoplasms, HCC patients are more susceptible to the effects of the COVID-19 pandemic because the hepatic injury[7] caused by SARS-CoV-2 could complicate the pre-existing hepatitis virus infection and cirrhosis in most HCC patients.

Due to the major treatment limitations and exposure risk created by the pandemic for patients with HCC, a general consensus amongst clinicians acknowledged that deviations from the standard of care are necessary, and specific international guidelines on HCC management have been drafted by the American Association for the Study of Liver Disease[8], the European Association for the Study of the Liver[9], the International Liver Cancer Association[10], the Asian Pacific Association for the study of the Liver[11], and the São Paulo Clínicas Liver Cancer Group Multidisciplinary Consensus Statement[12] (Table 1).

The aim of this review is to compare and summarize all these recommendations and guidelines, particularly in the diagnostic and therapeutic process of patients affected by HCC to provide clinicians with the best measures to be adopted in the framework of this ongoing pandemic.

SURVEILLANCE

As recommended by the American Association for the Study of Liver Disease and the European Association for the Study of the Liver, deferring HCC surveillance by 2–3 mo during the COVID-19 pandemic because of reduced radiologic capacity is likely

Table 1 Recommendations from main international liver society

	APASL	HC-FMUSP	EASL-ESCMID	ILCA	AASLD
Liver resection	Generally, liver resection with curative intent should not be delayed. However, in cases of high risk of decompensation or comorbidities, surgical intervention should be postponed or alternative therapy such as ablation should be adapted	The indications for surgical resection are the same as those during the prepandemic period and are as follows (18-21): In patients without cirrhosis: Solitary or oligonodular HCCs; in patients with chronic liver disease: Solitary tumors (regardless of size), preserved liver function (Child–Pugh A), and absent or mild portal hypertension (small caliber esophageal varices and platelets 100000/mm^3)			Can consider bridging locoregional therapy (TACE/TARE/SBRT), systemic therapy, or active monitoring if necessary to delay surgery
LT	LT for patients with poor short-term prognosis should not be delayed. Elective living donor transplantation may be suspended. In patients with complete response to bridging therapy on transplant list, transplantation may be suspended	LT is the treatment of choice for patients with early HCC (BCLC-A) and impaired liver function (Child–Pugh B/C), clinically significant portal hypertension, and those with early HCC who are not candidates for resection	Patients on the LT waiting list with decompensated cirrhosis are at high risk of severe COVID-19 and death following SARS-CoV-2 infection. We therefore recommend that LT centers aim to restore transplantation services following the peak of the COVID-19 epidemic wherever possible. In centers with ongoing resource limitations, LT should be prioritized for patients with poor short-term prognosis including those with acute liver failure, ACLF, high MELD score (including exceptional MELD points), and HCC at the upper limits of the Milan criteria	Unique considerations of COVID-donor derived infection and immunosuppression post-transplant. Consider cessation of LDLT (lower MELD) and delaying transplant in those with complete response	Limit the number of patients coming to clinic for transplant evaluations. Consider evaluating only patients with HCC or those patients with severe disease and high MELD scores who are likely to benefit from immediate liver transplant listing
Ablation	Ablation with curative intent should not be delayed. Ablation is an acceptable alternative to resection for cases of three or fewer tumors, each 3 cm or smaller, and of Child–Pugh class A or B liver dysfunction	Radiofrequency ablation can be performed in patients with very early (BCLC-0) or early (BCLC-A) HCC and have solitary nodules < 3 cm in size			Reserve for those with best chance of response (size < 3 cm) and can consider SBRT
Vascular intervention	Vascular interventions may be postponed because they are used as cytoreductive treatments in most cases. Vascular interventions should be suspended in cases of risk of decompensation or comorbidities that increase the risk of severe COVID-19	TACE/TAE: Can be performed in patients with solitary nodules > 3 cm in size as local disease control or as a "bridge treatment" to surgery			Consider TACE for single or multifocal HCC. Consider TAE, DEB-TACE or TARE instead of TACE and perhaps systemic therapy in some patients with large tumor burden
Radiation therapy	Radiation therapy for cases of symptom control or at low risk of progression may be postponed. However, radiation therapy for function- or life-threatening situations have to be treated without delay. The course of radiation	SBRT: Can be considered in patients who have contraindications to RFA or TACE/TAE			

	should be shortened when appropriate	
Systemic therapy	Oral tyrosine kinase inhibitors would be better than infusion regimens during the pandemic. The impact of immunotherapy on the course of COVID-19 is not known	Systemic therapy: May be used as a "bridge therapy" for surgery in patients with contraindications to other treatments

ILCA: International Liver Cancer Association; AASLD: American Association for the Study of Liver Disease; SBRT: Stereotactic body radiation therapy; COVID-19: Coronavirus disease 2019; TACE/TAE: Transarterial chemoembolization/embolization; LT: Liver transplantation; HCC: Hepatocellular carcinoma; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; LDLT: Living donor liver transplantation; MELD: Model for end-stage liver disease; ACLF: Acute-on-chronic liver failure; EASL-ESCMID: European Association for the Study of the Liver-European Society of Clinical Microbiology and Infectious Diseases; APASL: Asian-Pacific Association for the Study of the Liver society; RFA: Radio frequency ablation; TARE: Transarterial radioembolization; BCLC: Barcelona Clinic Liver Cancer; DEB-TACE: Drug-eluting bead transarterial chemoembolization; HC-FMUSP: São Paulo Clínicas Liver Cancer Group Multidisciplinary Consensus Statement.

safe. In patients with active COVID-19 infection, HCC surveillance should be postponed until they recover[13].

Although surveillance is important for early detection of HCC, risk varies between patients; in an environment with limited resources, risk stratification models[14-16] may be used even if they have not been fully clinically validated. This can be useful to identify patients who need to be prioritized for surveillance.

If ultrasound-based surveillance is not possible for extended periods of time because of lack of social distancing between operator and patient, blood-based biomarkers may be considered as an alternative strategy. Given the insufficient sensitivity and specificity of alpha-fetoprotein used alone[17], different biomarker panels have been proposed. The best evaluated is GALAD, which combines gender, age, and three biomarkers (alpha-fetoprotein, alpha-fetoprotein-lectin-reactive, and des-gamma carboxyprothrombin)[18].

HCC surveillance after curative therapy (surgery and ablation) is recommended and should be performed using cross-sectional imaging with multiphase abdominal computed tomography or contrast enhanced magnetic resonance imaging, the latter associated with noncontrast chest computed tomography[19].

A principle of maximizing the risk-benefit ratio should be taken for the surveillance of HCC and monitoring of patients who have received therapies for HCC. Prioritizing imaging resources to those patients at highest risk of incident HCC and recurrence following therapy, while prioritizing those patients who are eligible for an imminent liver transplantation (LT) is a judicious strategy to risk stratifying these patients.

INTERVENTIONAL RADIOLOGY

During the outbreak of the COVID-19 pandemic in early 2020, serious restrictions were applied in interventional radiology (IR) departments regarding the management of elective procedures. Resources and facilities were diverted to treat the large number of COVID patients, allowing limited opportunities for non-COVID-19 related procedures. Primarily urgent/emergency and carefully selected elective cases were performed in IR departments. This largely affected IR cancer care worldwide[20]. More specifically, for patients with HCC where meticulous coordination between different specialties and sequential various IR treatments (mainly percutaneous ablation and different types of transarterial embolotherapy) followed by strict imaging follow-up protocols are required to maintain an efficient therapy. The pandemic caused significant disturbances and negatively affected patient care[7].

There is consensus that HCC patients should continue to receive locoregional therapy during the COVID-19 pandemic, with choice of therapy discussed in a multidisciplinary format[8]. There is concern for serious COVID-19 infection in those receiving conventional transarterial chemoembolization (TACE) (with cytotoxic agents) because of systemic absorption with increased myelosuppression, and therefore the International Liver Cancer Association recommends other forms of locoregional therapy over conventional TACE (*e.g.*, bland embolization, drug-eluting bead-TACE, Y-90)[10]. Finally, consideration for earlier transition to systemic therapy could be considered in locally advanced HCC patients[21].

Several authors investigated the effect of the pandemic in HCC patients. In a study published by an Italian center, the authors reported that clinical practice adapted to minimize HCC patient exposure to the virus. As a result, a delay of treatment (transarterial procedures, thermal ablation, and systemic treatment) of ≥ 2 mo was noted in 26% of the patients during the COVID-19 outbreak compared with the same period in 2019[22]. Similarly, in a French multicentric study investigating patients from six referral centers of the metropolitan area of Paris (an area highly impacted by the COVID-19 pandemic), 21.5% of patients experienced a treatment delay > 1 mo in 2020 compared to 9.5% in 2019 ($P < 0.001$), although a significant difference in the modification in treatment strategy between the two periods was not noted[23]. Nevertheless, the impact of the COVID-19 pandemic on IR treatment outcomes in patients with HCC remains to be determined.

Generally, experts recommend avoiding treatment delays in HCC patients in order to decrease the risk of tumor progression, while patients with the highest chance for definite cure and those with the lower risk of developing severe complications should be prioritized for treatment. In an elegant review of current evidence, Chan *et al*[7] stratified the modifications of treatment required for HCC patients according to the stage of the disease[7]. The authors summarized that all patients should undergo multidisciplinary team evaluation using stricter selective criteria in order to identify those who would benefit the most from IR therapy. Optimally, patients could be referred in centers where IR services were less affected by the pandemic. In early-stage HCC, ablation was advised over surgical resection due to the limited availability of operating rooms, intensive care unit beds, and anesthetic facilities. In intermediate-stage HCC, TACE patient selection to identify patients that will benefit more could be performed using specific stratification scores, such as the Barcelona Clinic Liver Cancer (BCLC) stage B subclassification, the hepatoma arterial-embolization prognostic score, and the "beyond-up-to-seven" criteria. A personalized decision should be then taken regarding the number and frequency of TACE sessions, while in cases in which TACE therapy could not be appropriately followed due to unacceptable delays, systemic treatment or regular surveillance could be alternatively used.

SURGERY

Several guidelines have been proposed to clarify how to maintain standard of care of HCC in this pandemic era.

According to the European Association for the Study of the Liver- European Society of Clinical Microbiology and Infectious Diseases, listing for transplantation should be restricted to patients with poor short-term prognosis including those with acute/acute-on-chronic liver failure, high model for end-stage liver disease score (including exceptional model for end-stage liver disease scores), and HCC at the upper limits of the Milan criteria because transplantation activities/organ donations will likely be reduced in many countries and areas.

For patients already listed, SARS-CoV-2 routine testing should be performed before LT in both donors and recipients. Living-donor transplantations should be considered on a case-by-case basis. After LT, guidelines should be followed as usual trying to avoid as much contact as possible with medical staff.

In HCC patients, care should be maintained according to guidelines, including treatment and evaluation for LT[9].

A paper from the Italian Association of the Study of the Liver stated that the management of HCC has been negatively affected by the pandemic with, unfortunately, a reduction of surveillance and follow-up. Particularly, surgical procedures had been reduced or suspended in 44% of the centers. COVID-19 has also affected the liver transplant activity with a reduction in the number of performed transplants in 23% of the centers and interruption of the activity in less than 1%[24].

A multidisciplinary evaluation has been proposed by Santambrogio *et al*[25] with a treatment strategy adapted to the tumor size. Particularly, HCC less than 2 cm should be followed up monthly, those between 2 cm and 3 cm should be preferentially treated by local ablation (percutaneous or laparoscopic), while HCC > 3 cm require a surgical approach, preferably avoiding major hepatectomies and using the laparoscopic approach[25]. The Asian-Pacific Association for the Study of the Liver Society proposes that liver resection with curative intent should not be delayed. However, in cases of high risk of decompensation or comorbidities that increase risk of severe COVID-19, surgical intervention should be postponed or alternative therapy such as ablation should be adapted. Laparoscopic or robotic surgery during the pandemic may

contribute to decreased length of stay as compared with open surgery as well as minimizing the need for medical treatments. On the other hand, pneumoperitoneum, which is inevitable in laparoscopic or robotic surgery, may bring a higher risk of aerosol exposure to the surgeons and staff.

Transplantation should be decided on case-by-case basis. LT for patients with poor short-term prognosis, such as with high model for end-stage liver disease score and HCC at the upper limits of the Milan criteria are in high priority and should not be delayed. Those with compensated liver disease and within the lower limits of Milan criteria have medium priority and may be suspended to minimize the risk of the donor and the recipient. In patients with complete response to bridging therapy on the transplant list, transplantation may also be suspended until it can be performed safely with sufficient resources[11].

The International Liver Cancer Association suggests that surgical treatment should be offered to patients with low risk of decompensation and without comorbidities that increase the risk of severe COVID-19. If surgery is not possible, then alternative strategies should be performed (local ablation, TACE)[10].

The policy formulated by The Working Group Report of the Japan Association of Molecular Targeted Therapy for HCC recommends that patients not requiring emergency surgery, based on the macroscopic classification, degree of differentiation, and staging of the tumor, should be advised to avoid hospital admission by postponing surgery. The results of the surgery *vs* radiofrequency ablation trial indicate that radio frequency ablation, the less invasive option, should be proactively considered if there are ≤ three nodules each measuring ≤ 3 cm, which would shorten hospital stay[26]. If postponing surgical resection is considered, tumor growth should be suppressed using alternative outpatient therapy, such as bridging systemic therapy, with surgery rescheduled after carefully evaluating the risks and benefits of hospital admission in light of the COVID-19 pandemic[27].

The São Paulo Clínicas Liver Cancer Group Multidisciplinary guidelines state that surgery should be considered for patients with chronic liver disease and solitary tumors > 3 cm in size, to preserve liver function, with no clinically significant portal hypertension or other comorbidities, who are young, and with favorable locations for resection. This multidisciplinary group agrees with others that the living donor liver transplant program should be temporary suspended in order to preserve both donors and recipients.

They also consider delaying LT in patients with a complete response to "bridge therapies" and maintain close monitoring with imaging examinations to detect any recurrence. Patients with HCC who have significant liver dysfunction and/or viable tumors and have a high risk of losing eligibility for transplantation, especially those who do not respond to "bridge therapies" or present with tumor progression, should remain on the list for LT[12].

ONCOLOGY

During the COVID-19 pandemic, the medical community is experiencing a crisis due to lack of resources. But cancer patients represent a heterogeneous group that differ in prognosis, progression, and treatment. Oncologists have an important role to play for their patients in regard to resource reallocation[28]. In this era, the oncological approach to HCC treatment has been modified. Since February 2020, the first analysis related to SARS-CoV-2 infection patients demonstrated a higher risk of mortality between patients with cancer. The risk is due to the cancer but also to age, smoking history, and comorbidities[29]. In addition, patients with liver cancer appear to be more vulnerable because of underlying liver disease that can alternate both innate and adaptive immune responses[30].

The systemic treatment in advanced HCC (BCLC-C patients/Child Pugh-A liver function) is generally with multitargeted tyrosine kinase inhibitors characterized by daily oral administration (*e.g.*, lenvatinib, sorafenib). Not all countries have approved the combination of immune check point inhibitors and the antiangiogenic agent bevacizumab[31] with an intravenous administration. Oral tyrosine kinase inhibitors should be preferred over an infusion regimen during the pandemic to protect both patients and medical staff. The role of immunotherapy in SARS-CoV-2 infection is unknown. The use should be considered on a case-by-case basis[11].

In accord to the international and national guidelines during the COVID-19 pandemic, the telemedicine approach must be preferred[9,10]. Some recommendations suggest to rank in person patient visits as high, medium, and low priority. For

example, high priority patients are taking first and following line therapies, patients showing moderate or severe side effects to treatment, or with decompensated liver disease. In any case, a phone assessment is preferred[32].

If systemic therapy is ongoing, the use of telephone-based consultations should be preferred, enabling careful monitoring of patients without the need for frequent hospital visits over a short period of time[27]. The recommendation is to reduce the access in the hospital for a visit and improve telephone-based assessment with clinical evaluation and blood tests. In the case of in person visits, a phone call the day before the appointment should be made to consider the patients' general condition and the presence of any suspected symptoms (for example fever or rhinorrhea). In addition, at the hospital entrance temperature should be measured, hands should be disinfected, and the patients should be alone. Related to less complicated toxicities, such as hypertension, dermatological problems, and diarrhea, the management can be performed by community doctors after coordination with the patient's team[7].

For liver cancer clinical trials, regular visits should be maintained[33].

These modifications for clinical daily practice should be taken to reduce the risk of SARS-CoV-2 infection in patients with advanced HCC undergoing systemic treatment.

RADIOTHERAPY

During the COVID-19 pandemic, new guidelines have been introduced for the management of oncological treatments in radiotherapy (RT) departments[34,35].

Cancer patients are known to have a higher degree of fragility and therefore are inclined to complications of COVID-19 infection. Therefore, an appropriate risk-benefit assessment of each procedure should be considered as part of the treatment strategies for cancer patients during the pandemic[36].

RT is one of the cancer weapons, reporting a benefit in terms of overall survival and disease-free survival. By the introduction of advances in RT, providing high precision delivery while sparing at-risk organs, it is generally well tolerated with a median overall treatment time of 20-30 daily fraction. Furthermore, the use of hypofractionation or stereotactic RT treatments (SBRT) with few fractions (about 1-15) is preferred during the pandemic to reduce access times in hospitals.

In this context, SBRT treatment has a role as a noninvasive and effective therapy, for patients with HCC from the early stages to the most advanced stages. In general, the total dose on the tumor was 30-60 Gy in 3-5 fractions[34].

In early stage HCC, local therapy can be offered as a bridging treatment if surgery would be postponed or as a definitive therapy in the context of locoregional treatments, in the case of the inability to perform surgery[12,34].

In the case of intermediate stage HCC, SBRT has been proposed as an alternative option, particularly after an incomplete response to the previous TACE or for patients unfit for TACE[37]. Finally, in the case of locally advanced HCC with vascular invasion, RT with TACE has been shown to improve survival compared to TACE alone in patients with HCC[38]. In patients with advanced disease, the use of SBRT can reduce the time of suspension of systemic therapy if the patient requires palliative therapies to control symptoms derived from the disease[11,12,34].

In addition, the collaboration between 19 multidisciplinary liver specialists from high-volume liver malignancy academic centers in seven countries and five continents has resulted in a series of recommendations regarding the management of HCC patients in the era of COVID-19. According to the BCLC classification system, it is evident that RT and SBRT treatments are actively included in every stage of HCC[39].

When standard therapies are not available these recommendations can be considered: (1) BCLC-0 or BCLC-A: if a liver transplant or surgical resection is unavailable, consider bridging with locoregional therapies or surveillance. In this case SBRT 30–60 Gy/3–5 fractions; (2) BCLC-B: consider locoregional therapies, such as TACE, transarterial embolization, transarterial radioembolization, or SBRT 30-60 Gy/3-5 fractions or surveillance; and (3) BCLC-C: if patient has portal vein thrombosis and no extrahepatic disease, consider systemic therapy or a combination of TACE and radiotherapy (45 Gy in 15 fractions), SBRT in 3-5 fractions, or transarterial radioembolization; if patient has extrahepatic disease, use systemic therapy and/or palliative radiotherapy in a single 8 Gy fraction for symptomatic disease[12].

Using SBRT hospital access times are reduced compared to using standard fractionated or hypofractionated treatments (a max of six treatments for SBRT compared to fifteen or more in the other cases). Furthermore, the recommendations during the pandemic require the use of noninvasive immobilization systems, such as

active breathing and motion control management (breath-hold techniques, respiratory gating, abdominal compression) or free breathing treatment, according to the possibilities of each radiotherapy department, to mitigate respiratory movements avoiding the surgical implantation of liver fiducial markers during radiotherapy simulation phase and treatment.

It is fundamental for accurate triage to identify positive or suspected cases before and during treatment, and if the patient should test positive for SARS-CoV-2 during radiation therapy, then they may consider treatment suspension or the continuation of the same in a protected environment both in consideration of the patient's clinical condition and treatment purpose and in relation to the situation of each center to ensure a safe radiation treatment[36].

It is known, despite numerous phase II prospective and retrospective studies demonstrating SBRT safety and efficacy[40,41], that most guidelines in the management of patients with HCC limit the use of RT to patients who are not suitable or refractory to other locoregional treatments due to the lack of phase III trials. However, as discussed in the literature[42], it is anticipated that with more widespread clinical use of SBRT during the COVID-19 pandemic and the result of ongoing trials, there may be increased evidence to support the effectiveness of SBRT for HCC treatment.

CONCLUSION

The COVID-19 pandemic has strongly impacted the management of the oncological patient due to the reduction of inpatient beds and reallocation of nurses and doctors to the COVID departments that were rapidly developed in each hospital to face the pandemic. Despite this unexpected reorganization of hospitals, the necessity to continue to manage patients with HCC has required the continuation of multidisciplinary management while reducing the risk of COVID-19 negatively affecting the short and long-term oncological outcome. The main goals should be to optimize the risk/benefit balance and focus on patients with a more aggressive tumor. Flexibility has become mandatory in order to adapt to the different phases of the pandemic. The aim of these guidelines is to prepare physicians to manage the second and third wave of the COVID-19 pandemic.

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MINIREVIEWS

Human immune repertoire in hepatitis B virus infection

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Abstract

Hepatitis B virus (HBV) infection is a public health threat that affects 257 million people worldwide and can progress to liver cirrhosis, liver failure, and hepatocellular carcinoma. The HBV antigen- induced adaptive immune response plays an important role in HBV clearance. Immune repertoire sequencing (IRS) has been used to investigate the molecular mechanisms behind the immune system, find novel ways to treat HBV infection, and evaluate the genetic responses and immune characteristics of individuals infected by HBV or immunized by HBV vaccine. This review summarizes the human immune repertoire analysis methodology, and the application of the IRS in the prediction of HBV infection progression, treatment, and vaccination.

Key Words: Immune repertoire; T-cell receptor; B-cell receptor; Hepatitis B virus; Chronic viral infection; High-throughput sequencing

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Core Tip: A Hepatitis B virus (HBV) cure depends on activation of the anti-HBV adaptive immune system. Immune repertoire sequencing is a novel method to investigate all aspects of the human adaptive immune system. However, the immune repertoire in HBV infection is still not clear. We review the immune repertoire analysis methodology and provide a new insight into outcomes of HBV infection and vaccination.

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INTRODUCTION

Hepatitis B virus (HBV) is a hepatotropic, small, enveloped DNA virus that belongs to the Hepadnaviridae family and causes an acute or chronic infection in humans. The duration of infection is variable, ranging from 8 weeks to over 6 months. Chronic hepatitis B (CHB) infection is more common clinically in patients whose immune system fail to fight against HBV, and is characterized by a high HBV DNA load and HBsAg seropositivity. It can lead to advanced liver cirrhosis, liver failure, and hepatocellular carcinoma (HCC). CHB cannot be completely cured currently because of the existence of covalently closed circular DNA (cccDNA) in the cell nucleus, but it can be controlled by nucleos(t)ide analogs (NUCs) and interferon (IFN).

The immune response against HBV is the key to a cure, which involves both innate and adaptive immune responses. During acute infection, hepatocytes produce type 1 IFN, which inhibits viral packaging. The clearance of HBV is accompanied by asymptomatic or flulike symptoms[1]. Dysfunction and exhaustion of HBV-specific CD4+ and CD8+ T cells, decreased numbers and dysfunction of dendritic cells (DCs), nature killer (NK), and NKT cells are immune responses seen during CHB infection, as well as upregulated expression of immune molecules, including programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte antigen 4 (CTLA-4), and mucin domain -3 (Tim^3)[2-6]. Innate immune response are also downregulated during CHB infection, with dysfunction of toll-like receptors (TLRs)[7], which contribute to inducing a robust IFN response and suppressing HBV replication. In this review, we summarize the current state of knowledge of the immune repertoire and the relevance to understanding HBV infection (Table 1).

The immune repertoire includes the human T-cell receptors (TCRs) and B-cell receptors (BCRs) of the adaptive immune system. The generation of diverse TCRs expressed on the cell membrane of T lymphocytes and BCRs expressed by B lymphocytes maintain the balance of adaptive immunity and recognize the amounts of antigens. There are two types of T cells, αβT cells and γδT cells, according to their TCR genes. In αβT cells, the TCRs are composed of one alpha and one beta chain. In γδT cells, the TCRs consist of one gamma and one delta chain. The variable region of each chain consists of three complementarity-determining regions (CDRs), which are variable and determine the antigen specificity, and four frame regions (FRs). CDR1 and CDR2 are coded by variable (V) genes, and CDR3 is generated by random selection and recombination of variable (V), diversity (D), and joining (J) genes[8,9]. In all, the generation of diversity in TCR consists of a vast number of TCR gene segments recombinants, junction diversity, and alpha/beta or delta/gamma chain pairing during the development of T cells[10-12]. As for immunoglobulin (Ig) antibodies, BCR induces a Y-shaped protein composed of two large heavy chains encoded by the immunoglobulin gene heavy locus and two small light chains encoded by the immunoglobulin lambda locus (IGL) or immunoglobulin kappa locus (IGK). The heavy chain variable region is encoded by V, D, J gene families like those of T cells. The light chain is encoded by only V and J gene families. Before antigen stimulation, V(D)J recombination is mediated by several enzyme-like recombination-activating genes (RAG1/RAG2). Random additions and deletions of V-D and D-J (V-J for the light chain) joining regions contribute to the diversity of Ig[13,14]. The post antigen-stimulation response of Ig is more complicated, and includes somatic hypermutation, which is a programmed mutation process whereby changes are introduced to the nucleotide sequence of the immunoglobulin gene DNA during development[15]. The response also includes gene conversion, the asymmetrical segregation of genes during replication, which leads to the production of nonreciprocal recombinant strands and the apparent conversion of one allele into another[15]. Finally, class-switch recombination, B-lymphocyte gene rearrangement that results in substitution of the type of heavy chain constant region that is expressed and allows a change of the effector response while the antigen-binding specificity (*i.e.* the variable region) remains the same[15]. These three responses resulting in the functional differences of IgG, IgE, IgA, IgD, or IgM[16].

The ability of T cells and B cells to recognize and respond to their specific antigen is central to adaptive immunity. The fully functional antigen receptor complex with

Table 1 Immune repertoire investigation of hepatitis B virus infection

Number	Ref.	Patients	Amounts of samples	Repertoire	Sequencing reads	Tools
1	Deulofeut *et al*[59], 1997	2 volunteers	2	TCR repertoire	-	PRISM
2	Höhn *et al* [60], 2002	Healthy individuals immunized with recombinant hepatitis B surface antigen	7	TCR-VA and -VB repertoire in CD8+ T cells	-	FITC/PE
3	Soroosh *et al*[69], 2003	Nine healthy adult responders and six nonresponders	30	TCR beta chain variable gene		FRAGMENT MANAGER' software program
4	Yang *et al* [41], 2011	Forty-two patients with CSHB	42	TCR CDR3	-	SPSS 16.0 software
5	Xiong *et al* [44], 2014	18 patients with CSHB and 8 controls	26	TCR Vβ repertoire	-	DNAMAN software version 1.0
6	Han *et al* [52], 2015	Liver cancers and healthy adults	160	TCR CDR3	495708702	BLAST
7	Galson *et al* [62], 2015	Naïve group: n = 9; vaccine group: n = 9	108	B-cell repertoire	365863	R
8	Qu *et al* [37], 2016	4 CHB patients before and after HBeAg seroconversion	8	TCR Vβ repertoire	370210 to 685596	Homemade Perl script
9	Yang *et al* [55], 2016	12 HBeAg SC patients and 20 no HBeAg SC patients	96	TCRBV families	-	SPSS software version 19.0
10	Galson *et al* [63], 2016	9 healthy subjects	63	B-cell repertoire		R
11	Chang *et al* [70], 2016	4 pairs of HBeAg positive carrier and HBsAg negative non-carrier siblings	16	IgG immune repertoire	2.2 million	PEAR, Python, Numpy and SciPy, Ipython
12	Ma *et al* [64], 2017	3 healthy volunteers	6	IgM and IgG H chain CDR3 repertoires		IMGT/High V-Quest
13	Jiang *et al* [36], 2018	3 pairs of healthy identical twins and 7 pairs of chronic hepatitis B patients	20	CD8+ T-cell receptor beta (TCRβ) chains	50 million	MIXCR
14	Yan *et al* [49], 2019	6 patients with HBV-related ACLF and 6 controls	12	BCR CDR3 region	An average number of 12243860.30 in the control group and an average number of 1229965.30 in the ACLF group	GraphPad Prism software
15	Miyasaka *et al*[61], 2019	5 volunteers	30	T-cell and B-cell receptor repertoire	TCR β chain repertoire was 153151 before HB vaccination, 180093 after the second HB vaccination, and 129044 the third HB vaccination; BCR IgG heavy (H) chain repertoires were 106664 before HB vaccination, 126237 after the second HB vaccination, and 135663 the third HB vaccination	SPSS software package, Easy R (EZR) version 1.37, GraphPad software package
16	Shen *et al* [48], 2020	5 HBV-ACLF	20	TCR repertoire	163259321	MIXCR
17	Lian *et al* [57], 2020	20 CHB patients undergoing 1-yr ETV treatment (10 HBeAg SC patients and 10 no HBeAg SC patients)	60	T-cell repertoire	-	MIXCR

ACLF: Acute-on-chronic liver failure; BCR: B-cell receptor; CDR3: Complementary determining region 3; CSHB: Chronic severe hepatitis B; ETV: Entecavir; HBV: Hepatitis B virus; Ig: Immunoglobulin. SC: Seroconversion; TCR: T-cell receptor.

coreceptors and costimulatory receptors on T cells and B cells has not only exquisite

antigen specificity but also different signaling pathways (Figure 1). The T-cell receptor complex consists of an antigen-binding TCR α and TCR β heterodimer associated with CD3 that has four signaling chains (two ε, one δ, and one γ), as well as a homodimer of ζ chains. Each CD3 chain has one tyrosine-based immunoreceptor activation motif (ITAM); each ζ chain has three. After a T cell has detected its specific antigen, phosphorylation of tyrosine residues in the ITAMs of the TCR enables binding of the cytosolic tyrosine kinase-zeta chain of the TCR-associated protein kinase 70 (ZAP-70), followed by the CD4 and CD8 coreceptors, which bind to major histocompatibility complex (MHC) class 2 molecules and class 1 molecules. Activated ZAP-70 leads to membrane recruitment of phospholipase C-γ (PLC-γ), which initiates three important signaling pathways that involve activation of nuclear factor of activated T cells (NFAT), nuclear factor kappa B (NF-κB), and activator protein-1 (AP-1). Antigen detection thus results in the differentiation and proliferation that characterize the immune response[17,18]. The BCR complex includes cell-surface immunoglobulin with one each of the invariant signaling proteins, Igα and Igβ, each of which has a single ITAM in their cytosolic tails that enables signal initiation after the BCR binds to an antigen. The logic of BCR signaling is similar to that of TCR signaling, but some of the signaling components are specific to B cells. When BCRs have bound a multivalent antigen, which cross-links them, three protein tyrosine kinases of the Src-family, Fyn, Blk, and Lyn, are activated and phosphorylate the ITAM tyrosine residues, which creates binding sites for the cytosolic protein, kinase spleen-associated tyrosine kinase (Syk). Syk then phosphorylates and activate the enzyme PLC-γ, which then initiates signaling pathways just as occurs with TCRs[19,20].

IMMUNE REPERTOIRE ANALYSIS METHODOLOGY

High-throughput sequencing (HTS) has increased the range, complexity, sensitivity, and accuracy with a great increase in the scale of operation, the number of nucleotides, and the number of copies of each nucleotide sequenced[15]. Investigation of the immune repertoire has thus become more effective, convenient, and less costly. The depth and amount of sequencing data obtained from of disease-specific TCR or BCR clones provide investigators with a great chance to identify individualized and common clonality during HBV infection. There are five phases of immune repertoire analysis starting with cell isolation or tissue collection (Figure 2). After collecting patient samples (cells and/or clots), DNA or mRNA is extracted, purified, and sequenced. DNA, is more suitable for calculating the proportions of antigen-specific T or B cells and for studying the functional/phenotypic evolution of specific TCR/BCR clonotypes. Therefore, it is recommended to choose mRNA to study cell function and activation[21].

It is important to complete the library preparation and amplification as that affects the accuracy of sequencing data. Two next-generation sequencing (NGS)-based amplification methods are currently applied. Multiplex PCR is the most convenient and straightforward approach for DNA samples. Because the V segment is highly variable, multiplex PCR incorporates V and J gene [constant (C) gene for mRNA] specific multiplex primers to amplify the full recombined variable region or CDR3 region of TCR/BCR gene[22,23]. PCR with the 5′ RACE method for rapid amplification of cDNA ends, which only works with mRNA, but can theoretically avoid V gene bias. RNA is reverse transcribed with a gene-specific primer targeting a known 3′ end sequence. The 5′ end of an unknown sequence is amplified with a synthetic oligonucleotide[24,25]. Other methods like anchored multiplex PCR (AMP) introduced by Zheng *et al*[26] and TCR ligation-anchored-magnetically captured PCR (TCR-LA-MC PCR) introduced by Ruggiero *et al*[27] are designed to avoid amplification bias and sequencing sensitivity.

The fourth phase of immune repertoire is sequencing with HTS, which has been used since 2009. The most widely used HTS platforms are the Roche 454 sequencing system and the Illumina HiSeq platform. The Roche 454 sequencing system was the first HTS platform. It provides an average 500 bp read length per run and sequences millions of molecules per repertoire[28]. The Illumina HiSeq platform provides a shorter read length but significantly higher read throughput and a lower cost per read compared with the Roche 454, which cater to the demand of deep sequence of the complex immune repertoire[20,29].

The fifth phase is analyzing the TCR and BCR sequence data with appropriate bioinformatic tools. Many computational tools are available to analyze HTS data including IMGT/High V-QUEST[30], new IgBLAST[31], Decombinator[32], pRESTO

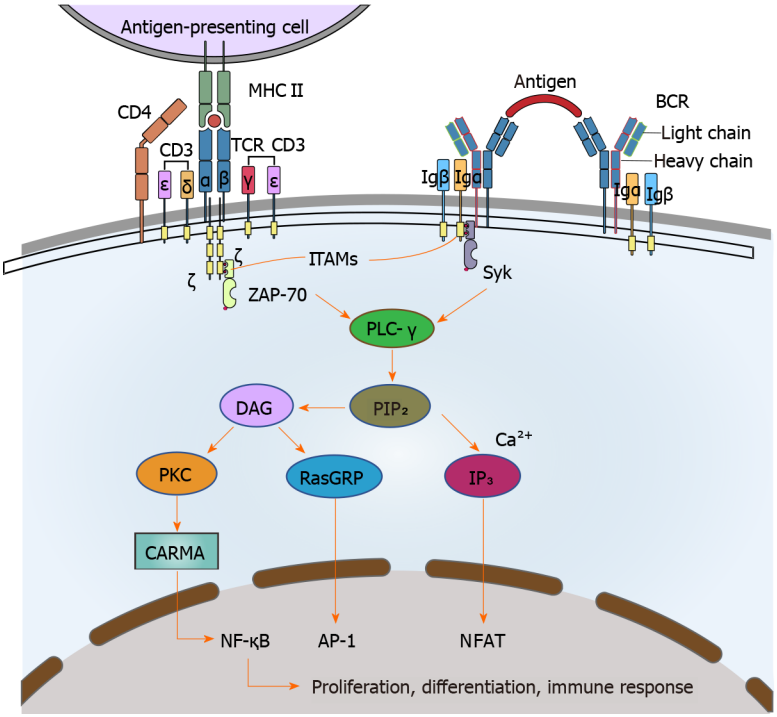


Figure 1 T-cell receptor and B-cell receptor structure and signaling pathways. T-cell receptor and B-cell receptor complexes include both variable antigen-recognition proteins and invariant signaling proteins. Phosphorylation of the ITAMs in CD3 ε, γ, δ, and the ζ chain enables them to bind the cytosolic tyrosine kinase ZAP-70, which in turn recruits and activates PLC-γ. Activated PLC-γ cleaves PIP_2 to yield DAG and IP_3. IP_3 increases intracellular Ca^{2+} concentration, activating calcineurin, a phosphatase that then activates an NFAT transcription factor. DAG recruits PKC to activate CARMA, which leads to activation of NF-κB and recruits RasGRP, which activates AP-1. These three important signaling pathways activate transcription factors in the nucleus, including NF-κB, NFAT, and AP-1, which result in cell differentiation, proliferation, and immune response. AP-1: Activator protein-1; CARMA: Caspase recruitment domain family, member 14 protein; DAG: Diacylglycerol; IP_3: Inositol trisphosphate; ITAM: Immunoreceptor tyrosine-based activation motif; NFAT: Nuclear factor of activated T cells; NF-κB: Nuclear factor kappa B; PIP_2: Phosphatidylinositol bisphosphate; PKC: Protein kinase C; PLC-γ: Phospholipase C-γ; RasGRP: RAS guanyl releasing protein; Syk: Spleen-associated tyrosine kinase; ZAP-70: Zeta chain of T-cell receptor-associated protein kinase 70.

[33], and MiXCR[34]. These bioinformatic tools can be used for VDJ gene assignment, CDR and FR annotation, CDR3 length identification, insertion and deletion analysis, and mutation spectrum analysis. What's more, the international immunogenetics information system (ImMunoGeneTics database) also provides important information about specific BCR and TCR V, D, J, and C genes[35].

CHARACTERISTICS OF THE IMMUNE REPERTOIRE IN HBV INFECTION PROGRESSION

Chronic HBV infections can persist with an asymptomatic carrier status or as chronic hepatitis B (CHB) that can progress to chronic severe hepatitis B (CSHB), cirrhosis, HBV-related acute-on-chronic liver failure (HBV-ACLF), and HCC. The progression of HBV infection is associated with the immune response, especially the adaptive immune response. The characteristics of the immune repertoire in HBV infection progression involve both TCRs and BCRs. Hepatitis B e antigen (HBeAg) seroconversion is an important step toward achieving a CHB cure, which appears to be dependent on an immune response to clear the virus. Jiang *et al*[36] analyzed $CD8^+$ T-cell receptor beta (TCRβ) chains in seven pairs of monozygotic twins with CHB and three healthy control pairs by HTS. Six pairs were infected with HBV during childhood; four of the six pairs had the same clinical outcomes. A high level of similarity in the TCR repertoire of each pair was found in average TCR Vβ segment expression and the frequency of the CDR3 pattern and skewed or oligoclonal clonotypes. Notably, the detailed CDR3 pattern and frequency were related to disease prognosis. There was an increased abundance of immunodominant clonotypes in patients with HBV antigen seroconversion[35]. Analysis of the TCR β chain repertoire in PBMCs from four CHB patients with HBeAg seroconversion demonstrated that TRBVβ12-4, Vβ28, Jβ2-1, V7-2-01-J2-1, V12-4-J1-1, and V28-1-J1-5 were associated with the development and treatment of CHB. No significant changes were observed following seroconversion

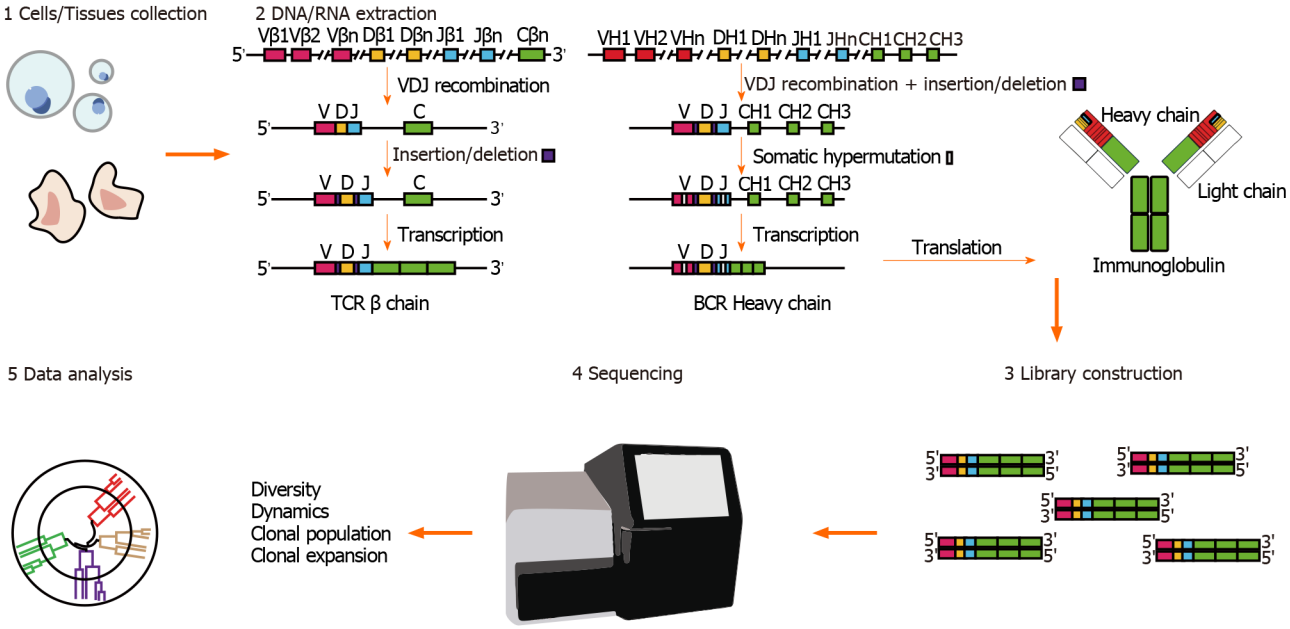


Figure 2 Workflow for immune repertoire sequencing. There are mainly five sections in immune repertoire analysis. 1: Cell/tissue collection. 2: DNA/RNA extraction. The figure illustrates the gene recombination and transcription of T-cell receptors (TCRs) and B-cell receptors (BCRs). The V, D, and J gene segments of TCRs and BCRs undergo somatic rearrangement and gene insertion/deletion before transcription to generate highly variable CDR3 regions, which are then translated as TCRs and immunoglobulins. 3: Library construction by next-generation sequencing methods are used to complete the library preparation and amplification. 4: Data sequencing. 5: Data analysis. BCR: B-cell receptor; CDR3: Complementary determining region 3; TCR: T-cell receptor.

[37].

CHB patients who develop CSHB have more than 50% mortality, and the rate is related to significant increases in the levels of CD8+ and nonspecific T cells[38,39]. Analysis of TCR Vβ diversity in peripheral CD4+ and CD8+ T lymphocytes obtained from 18 patients with CSHB found that CD8+ T cells play a major role in the pathogenesis of CSHB[40]. CDR3 spectratype analysis showed predominant expression of TCR Vβ5, Vβ7, Vβ9, Vβ12, and Vβ18 families in the CD8+ T cell subsets of CSHB patients. In addition, JB1S1 and JB2S7 region genes were present at a high frequency. Furthermore, three conserved amino acid motifs were identified, including GSF, LF and GS, which may be involved in binding to HBV-specific antigens[40]. In contrast, TCR Vβ7 and Vβ11 were more frequently expressed in the PBMCs and CD8+ subsets of CSHB patients, possibly because of differences in the human leukocyte antigen (HLA) types and HBV genotype variants. Interestingly, the two CDR3 amino acid sequences had conserved BV11, AGEL or VYNEQ and BV7, QDSVTTGAQ motifs, and the CSHB patients who expressed the "AGEL" TCRBV11 CDR3 amino acid sequence had better short-term responses than patients expressing the "VYNEQ" TCRBV11 CDR3 sequence[41]. Immunostaining of liver biopsies from untreated CHB patients found the portal-periportal CD4+ and intralobular CD8+ cell frequencies were increased in severe hepatitis, which indicated that the CD4+ T cells were also active in the liver micro-immune environment[42].

CHB can progress to HBV-related HBV-ACLF, which is the most common type of liver failure. It is characterized by rapid deterioration of liver function, coagulopathy, and subsequent multiple organ failure with a 28-d mortality in the Asia-Pacific and African regions[43,44]. Recent studies indicated that patients with HBV-ACLF had more IL-17-producing CD8+ T (Tc17) cells than patients with CHB, a decrease in CD4+ and CD8+ T cells, or an increase in regulatory T cells (Tregs) in HBV-ACLF patients [45-47]. Shen *et al*[48] studied the dynamic changes of TCR repertoires in patients with HBV-ACLF and demonstrated that there was a significant decrease in the diversity of CD8 T-cell repertoire that was positively correlated with a reduction of the Model for End-Stage Liver Disease score. CD8 TCRβ repertoire diversity may be a potential predictive marker of disease outcome[48]. Yan *et al*[49] analyzed BCR heavy chain CDR3 sequences from patients with HBV-ACLF and control subjects and found that clonal expansion was more extensive in the ACLF patients and the distribution ratios of the V, D, J and VJ combinations revealed differential expression, with six upregulated genes and 19 downregulated genes in the ACLF patients. ACLFspecific BCR CDR3 sequences hold future therapeutic promise for HBVACLF[49].

Table 2 Specific TRB family and clinical significance

TRB family	Clinical significance
TRBVβ2	Development and treatment of CHB
TRBVβ3	HBeAg seroconversion
TRBVβ5	Severity of CHB
TRBVβ7	Development and treatment of CHB, severity of CHB
TRBVβ9	Severity of CHB
TRBVβ11	HBeAg seroconversion, severity of CHB
TRBVβ12	Development and treatment of CHB, HBeAg seroconversion, severity of CHB
TRBVβ14	HBeAg seroconversion
TRBVβ18	Severity of CHB
TRBVβ20	HBeAg seroconversion
TRBVβ24	HBeAg seroconversion
TRBVβ28	Development and treatment of CHB

CHB: Chronic hepatitis B; HBeAg: Hepatitis B e antigen.

HBV is the most common cause of HCC, with an estimated prevalence of 44%-55% of HCC cases worldwide[50]. The mechanism underlying HBV-related HCC and other HBV-related liver cancers is not clear. Some studies have shown that the HBV x gene promotes cell cycle progression, inactivates negative growth regulators, and inhibits the expression of the p53 tumor-suppressor gene[51]. The adaptive immune system also plays an important role in carcinogenesis. An investigation of TRB V usage in HBV-associated HCC found that the T-cell repertoires of HCC, intrahepatic cholangiocarcinoma, and mixed hepatocellular and cholangiocellular carcinoma tumors and adjacent tissues were significantly different ($P < 0.01$). The highly expanded clone ratio in blood samples from liver cancer patients differed significantly from those in the blood of healthy adults and hepatitis patients. The results suggest that comparison of the T-cell repertoires of tissue and blood could be used to distinguish liver cancer patients from healthy adults and from hepatitis patients[52].

APPLICATION OF IMMUNE REPERTOIRE ANALYSIS IN HBV THERAPY

Current treatment of CHB is limited to nucleos(t)ide analogs, such as entecavir (ETV), tenofovir disoproxil fumarate (TDF), tenofovir alafenamide (TAF), and others, which block DNA synthesis; and interferon-α, which direct suppresses viral RNA and protein production in infected cells and inhibits HBV DNA replication. It also activates NK cells and T cells that target HBV-infected cells[2]. Studies that evaluated HBV treatment found differences in the antiviral effect that were associated with the adaptive immune response. Yan *et al*[53] reported that telbivudine reduced HBV DNA levels and downregulated the proportion of circulating Tregs. A reduction in the Treg proportion was observed in CHB patients during TDF treatment[54].

A clinical trial including 12 patients with HBeAg seroconversion and 20 without seroconversion who were treated with 300 mg TDF daily for 96 wk found a T-cell receptor beta-chain variable (TCRBV) family that was associated with HBV DNA levels in the seroconversion group[55]. Six TCRBV families (BV3, BV11, BV12, BV14, BV20, and BV24) were more prevalent than the other TCRBVs in the seroconversion group. BV12, BV15, and BV22 were predominant in the patients who had not seroconverted. In addition to the Treg frequency and alanine aminotransferase (ALT) level, the Treg profile and TRBVs were associated with HBeAg seroconversion and may be potential predictors of HBeAg seroconversion and treatment outcome in CHB patients [55,56]. A recent TCR repertoire study by Lian *et al*[57] in 20 CHB patients undergoing 1-year ETV treatment found that the diversity of TCRβ repertoires was decreased in the 10 HBeAg- seroconverted patients but increased in the 10 patients without HBeAg seroconversion. The number of unique TCRβ clonotypes was positively correlated with the ALT or HBV DNA level in seroconverted patients, which indicated that the

TCR repertoire along with ALT or HBV DNA level may be a biomarker to predict HBeAg seroconversion during and after antiviral treatment[57]. The TRB family specific to HBV infection and its clinical significance are shown in Table 2.

APPLICATION OF IMMUNE REPERTOIRE ANALYSIS IN HBV VACCINATION

The immune repertoire plays an important role in HBV vaccine response. The HBV vaccine consists of recombinant HBsAg, an aluminum hydroxide adjuvant, and/or a virus-like particle that stimulates T-helper (Th2) cells to produce IL-4 to promote the production of anti-HBsAg antibodies[58]. The human TCR repertoire response to HBsAg is oligoclonal, involves multiple TCRBV families, and has individual specificity [59]. Analysis of the TCR-VA and -VB repertoire in CD8+ T cells from individuals immunized with recombinant HBsAg detected monoclonal TCR transcripts exclusively in CD8+, but not in CD4+ T cells[60]. TCR β chain CDR3 repertoire diversity increased, and the BCR IgG H chain CDR3 repertoire diversity decreased, indicating that diversity changes may be associated with a better response to the HBV vaccine [61]. An investigation of the BCR repertoire by Galson *et al*[62,63] found that vaccine-specific BCR sequence clusters expanded after each of three sequential vaccine doses. Additionally, many vaccine-specific BCR clusters appeared to largely derive from previously activated cross-reactive B cells that had low affinity for the vaccine antigen, and subsequent doses were required to yield higher affinity B cells[62,63]. Analysis of the IgG and IgM heavy chain CDR3 repertoire before and after immuni-zation with recombinant HBV vaccine, found the diversity of IgG heavy chain CDR3 repertoires was 1/6 of IgM on average[64]. Moreover, the mechanism of high frequency CDR3 generation was associated with the maturation of IgG affinity[64].

About 5% to 10% of healthy adults fail to produce protective levels of anti-hepatitis B surface antibodies after vaccination with recombinant anti-HBsAg vaccines[58,65]. In "nonresponders," the mechanisms that contribute to the lack of humoral immune response to HBsAg include defective in Th1- and Th2-specific responses, dysfunction of antigen-presenting cells, immunologic tolerance, and too few HBsAg-specific B cells and T cells[66-68]. Study of the immune repertoire could provide a new perspective to overcome obstacles by understanding the adaptive immune profile to HBV vaccination. Differences in the BV5S2-3 gene family in CD4+ T cells and several TCR BV genes, such as BV12 in the CD4+ population and BV24 in the CD8+ population, may explain the unresponsiveness to recombinant HBsAg vaccine[69].

CONCLUSION

IRS has demonstrated its potential in advancing our understanding of the progression of HBV infection, antiviral treatment, and vaccination. Ongoing study of immune repertoires in the field of HBV infection, overcoming technical problems, and increased sharing of sequencing data, the reporting of interesting clinical discoveries will increase.

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MINIREVIEWS

Emerging applications of radiomics in rectal cancer: State of the art and future perspectives

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Abstract

Rectal cancer (RC) is the third most commonly diagnosed cancer and has a high risk of mortality, although overall survival rates have improved. Preoperative assessments and predictions, including risk stratification, responses to therapy, long-term clinical outcomes, and gene mutation status, are crucial to guide the optimization of personalized treatment strategies. Radiomics is a novel approach that enables the evaluation of the heterogeneity and biological behavior of tumors by quantitative extraction of features from medical imaging. As these extracted features cannot be captured by visual inspection, the field holds significant promise. Recent studies have proved the rapid development of radiomics and validated its diagnostic and predictive efficacy. Nonetheless, existing radiomics research on RC is highly heterogeneous due to challenges in workflow standardization and limitations of objective cohort conditions. Here, we present a summary of existing research based on computed tomography and magnetic resonance imaging. We highlight the most salient issues in the field of radiomics and analyze the most urgent problems that require resolution. Our review provides a cutting-edge view of the use of radiomics to detect and evaluate RC, and will benefit researchers dedicated to using this state-of-the-art technology in the era of precision medicine.

Key Words: Computed tomography; Magnetic resonance imaging; Radiomics; Rectal cancer; Clinical applications; Overall survival

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Core Tip: Radiomics has exhibited significant potential for risk stratification of rectal cancer and has yielded excellent performance in response assessment of neoadjuvant radiochemotherapy. While the past 3 years has witnessed an exponential growth of the

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field, research on radiomics remains in its infancy and is constantly evolving. More rigorous analyses are emerging, and improvements in bias reduction techniques accompanied with multicentric studies will hopefully enable more robust and generalizable models. Here, we review recent updates on the use of radiomics based on computed tomography and magnetic resonance imaging in the detection and evaluation of rectal cancer.

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INTRODUCTION

Colorectal cancer (CRC) is ranked third among the most common cancers worldwide and accounts for nearly one-tenth of cancer-related deaths globally[1,2]. Rectal cancer (RC) comprises 27%-58% of all CRCs[3]. The development of treatment strategies and the revision of multidisciplinary treatment approaches such as local excision, total mesorectal excision (TME), and neo-adjuvant radiochemotherapy (nCRT) have decreased local recurrence and distant metastasis rates in recent decades[4,5]. However, accurate pretreatment tumor staging by imaging remains essential for precise decision-making[6].

Traditional medical imaging is routinely used in initial diagnosis of RC and has played a critical role as a non-invasive tool during follow-up[7]. According to the 2018 European Society of Medical Oncology (ESMO) Guidelines, high-resolution magnetic resonance imaging (MRI) is the standard staging modality for RC and has exhibited superior performance for tumor staging when compared with digital rectal examination, computed tomography (CT), and endoscopic ultrasound[8]. CT is mainly used for local lesions due to its inherent traits of low soft-tissue contrast, which limits the accurate approximation of T stage. Even in T4 lesions with gross invasion of adjacent organs, false-positive cases may occur. Therefore, CT is typically employed primarily for the detection of metastases. CT has faster acquisition time and is more practical than MRI, as it is more widely available[9]. However, both CT and MRI have restricted resolution in clinical applications.

Radiomics has emerged with consistently developing methodology and promising results[10-12]. Radiomics is a method that enables quantitative extraction of radiomics features that cannot be captured by visual inspection from available radiological images[13]. In the past few years, an increasing number of studies have evaluated abdominal radiomics models in different oncological scenarios and reported impressive performance for evaluating tumor biological behaviors, prognosis, and prediction of therapeutic responses[14-16]. Radiomics has been validated as a novel approach for improved characterization of tumor subtypes and the lesion microenvironment[17,18]. By making use of the medical images and clinical data, radiomics models have the potential to provide more detailed information to tailor individualized treatment scheme and patient management[19-21].

The purpose of this review is to describe and summarize the recent advances in clinical applications of radiomics based on CT and MRI, to highlight the potential role of radiomics in disease evaluation and clinical decision-making of RC, and to discuss the current limitations and possible optimization directions in the future.

RADIOMICS WORKFLOW AND METHODOLOGICAL ADVANCES IN RC

The concept of radiomics was first developed by Lambin *et al*[22] in 2012. Radiomics is defined as a research method that includes quantitative data extraction from multimodality medical images, analysis, and modeling of high-dimensional medical image features to explore relationships with clinical outcomes[11,13]. Related research can be divided into five stages: Data acquisition and analysis, medical imaging segmentation, feature extraction and selection, clinical target-oriented modeling, and research quality evaluation. The workflow is presented in Figure 1.

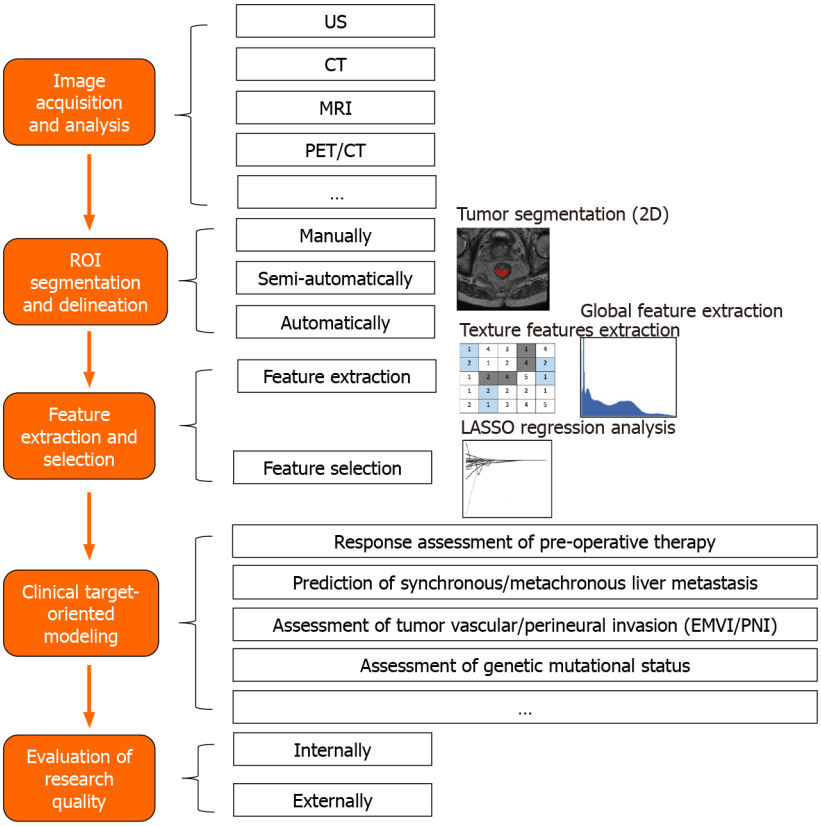


Figure 1 Workflow of radiomics applied in rectal cancer. US: Ultrasonography; CT: Computed tomography; MRI: Magnetic resonance imaging; PET: Positron emission tomography; ROI: Region of interest; EMVI: Extramural venous invasion; PNI: Perineural invasion.

Data acquisition and analysis

The radiomics workflow begins with the determination of the region of interest (ROI), which depends on the specific clinical problem that requires resolution. Data used in radiomics studies may be retrospective or prospective and single-center or multi-center. ROIs are determined by specific targeted research; therefore, researchers should first define the clinical problems to solve. Here, we consider RC as an example, whereby primary tumors are analyzed and related to existing treatment outcomes such as survival or recurrence. The analysis of lesions and normal tissues may affect the treatment strategies. *Via* the establishment of a large image database, a large body of imaging data is stored to generate an integrated medical and health care network. Radiologists should be cognizant that imaging protocols may not always be standardized, and variability exists among medical images and institutions. In this regard, the recommendations of the Image Biomarker Standardization Initiative may help to reduce the variability in image pre-processing prior to analysis[23].

ROI segmentation and delineation

Normalizing the original image is essential because data results may be affected by different machines or different parameters during collection. The process of ROI segmentation in radiomics can be performed either manually, semi-automatically, or automatically with software. Each approach has advantages and disadvantages. Manual segmentation is more precise in some occasions (*e.g.*, delineating the RC bed after nCRT) but has lower repeatability. Automatic segmentation depends on algorithms, which are efficient and may help to eliminate subjective errors[24]. To date, a mature automatic segmentation algorithm for RC is lacking. According to our PubMed search results, most radiomics studies on RC applied manual segmentation in which the segmentation is performed by radiologists to annotate the location and precise boundary of the ROI. Itk-snap software (www.itksnap.org) is used extensively for segmentation. Figure 2 shows the segmentation of a rectal tumor using Itk-snap software. Given that subjective bias may occur, segmentation may be inconsistent. At present, several steps are enacted to minimize bias, including the involvement of different medical professionals, multiple segmentation in different respiratory cycles, and adding noise to segmentation[25].

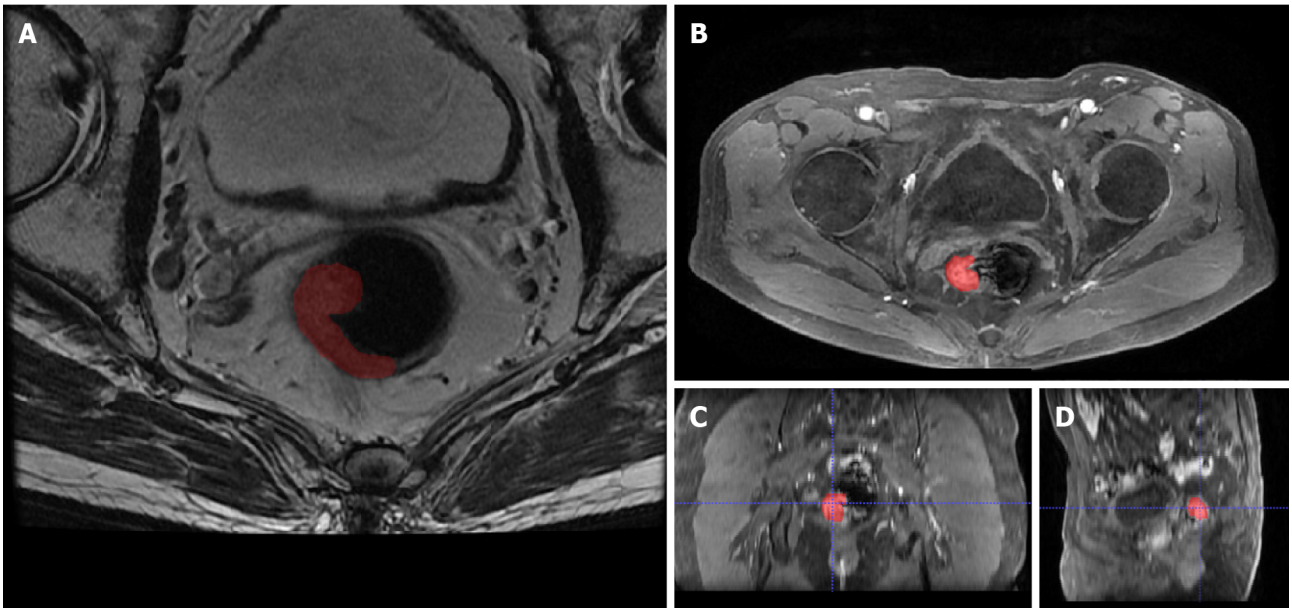


Figure 2 Segmentation of a rectal tumor with Itk-snap software. A: Example of tumor segmentation using Itk-snap software (www.itksnap.org) on axial plain magnetic resonance image; B-D: Axial (B), reconstructed coronal (C), and sagittal (D) contrast-enhanced magnetic resonance images in the venous phase in a 72-year-old man with rectal cancer.

Feature extraction and selection

Following the evaluation of the consistency of feature calculations across researchers, the initial selection of high-level feature sets is performed, and feature values that are sensitive to subjective factors are ultimately retained. Parmar *et al*[26] used the "IRR" (evaluator reliability) package in R software to evaluate the consistency of features extracted from ROIs, whereby features with a consistency coefficient less than 0.8 would be deleted.

The key to radiomics is the extraction of high throughput features that are difficult to depict by observers using mathematical algorithms from ROI segmentation directly. These features of essential value can either be directly extracted from original medical images or after applying a transformation or filter method. The process can be performed using different tools (*e.g.*, PyRadiomics, Texrad, MaZda, and others) which are readily available as open-source software. Image filtration enhances the edge qualities and removes interference by noise, thus permitting the collection of more information on spatial locations of features. At present, the main extraction methods are improved based on the method published by Aerts *et al*[27] in 2014, according to which radiomics features are divided into machine-learning-based features and deep learning features. There are several commonly used subgroups of the former, such as shape features (describing the geometric attributes of the ROIs), histogram-based features (capturing the first-order statistical characteristics of rectal lesions), and texture features (describing the granular textural pattern of the ROIs). Commonly used engineered features according to "order" are presented in Table 1. Recently, a set of 169 standardized radiomics features was published, which enabled the verification and calibration of different radiomics software[23].

Given that the size of the data sample is relatively small compared with features, this may result in over-fitting and it may be time-consuming to include all features in the classification. Feature selection is a necessary step to obtain features that are closely related to the target results in radiomics analyses. Typically, features extracted by radiomics are high-dimensional in a dataset, and a large proportion of features may not be useful for the task; hence, unstable features should be excluded to retain the most important features and prevent over-fitting. The main methods applied in feature selection can be divided into univariate or multivariate, based on whether the association among features can be considered to contribute towards a specific outcome. In machine-learning, the most commonly used feature selection algorithms include least absolute shrink and selection operator regression, minimum redundancy maximum correlation, recursive feature elimination, and principal component analysis [28].

Table 1 Commonly-used radiomics feature categories according to "order"

"Order"	Paraphrase	Categories	Description
First-order	Based on an intensity histogram of pixel values, providing no information on neighboring interactions or the spatial distribution	Mean	Average intensity of the pixels
		Skewness	Asymmetry of histogram
		Kurtosis	Magnitude of pixel distribution
		Entropy	Irregularity of the structure
Second-order	Considers the spatial relationship between 2 pixels	Grey level co-occurrence matrix (GLCM)	Frequency of specific gray values along a distance or direction
		Grey level run Length Matrix (GLRLM)	Length of consecutive pixels or voxels with the same grey values in a specific direction
		Grey level size zone matrix (GLSZM)	Length of consecutive pixels or voxels with the same grey values in all directions
Superior-order	Describes the neighborhood gray difference matrices and the relationship between 3 or more pixels	Neighboring gray tone difference matrix (NGTDM)	Describes the sum and average grey levels of discretized voxels in planes
		Gray level dependence matrix (GLDM)	Describes the coarseness of overall texture by evaluating the grey levels between a voxel and the neighborhood in 3 dimensions

In reference to "order", it is defined as the number of stages required to obtain the quantitative information in a model.

Clinical target-oriented modeling

Radiomics feature analysis and modeling involve the establishment of a prediction model based on selected imaging characteristics. The model generally encompasses clinical, biological, and occasionally genetic information. Commonly used models include logistic regression, support vector machine (SVM), and random forest[13]. In radiomics analysis, machine-learning algorithms are typically required to establish a classification or prediction model to obtain reliable results instead of relying solely on single factor analysis. The Cox proportional risk model is usually employed as a survival analysis model. Each modeling method has its own limitations. For example, feature independence, feature discretization, and network configuration dependence should be considered in logistic regression, Bayesian networks, and deep learning, respectively.

In the process of model-building, researchers can employ different software tools. R language contains software packages for data-mining and modeling. Clinicians and radiologists can operate various components *via* a graphical interface, compare different modeling algorithms, and identify the most effective way to resolve clinical issues. SPSS modeler is a commercial data-mining software tool with functions encompassing almost all data-mining procedures. This software possesses a graphical operation interface and automatic modeling function, which permits management of large amounts of data and provision of steady data-mining models. Other software for modeling, data-mining, and analysis, such as Weka (based on JAVA) and B11 (used in combination with Mazda), may also be employed. These software tools contain various modeling algorithms, including artificial neural network, k-nearest neighbor, K-means, hierarchical clustering, and similarity-based clustering methods.

Evaluation of research quality

For a high-quality radiomics study, it is essential to try different types of modeling methods and to choose the best algorithms. Moreover, all the models should be validated internally and externally (multicenter validation would be better if permitted)[29]. Validation is an indispensable part of complete radiomics analysis, because the value of the unverified evaluation model is limited. Except for validation of the model, quality assessment should also be conducted to ensure reproducibility in a radiomics study. A prediction model is appropriate for clinical decision-making only when a standardized evaluation of its performance is accomplished.

Radiomics based on deep learning

Deep learning is defined as a deep neural network structure based on a broad spectrum of algorithms, and the frameworks permit machines to learn highly complex mathematical models for data representation and can subsequently be used to perform

accurate data analysis. Radiomics algorithms based on deep learning have developed substantially in recent years[30,31]. Three types of deep learning models are commonly used in medical imaging: Convolutional neural networks (CNNs), generative adversarial networks, and sparse auto encoders. Deep learning-based radiomics performs learning procedures *via* convolutional operations and CNN structures. Compared with traditional radiomics, convolution operation has strong feature extraction abilities. The neural network structure can flexibly extract different task-related features by changing the convolution kernel and modifying the structure, thereby enabling a more targeted approach. Different features can be obtained by adaptively changing the convolution kernel. For example, convolution and Laplacian kernels can extract high-frequency features, while the Gaussian kernel can propose low-frequency features. For this reason, the deep learning approach exhibits superior feature extraction by extracting and selecting supplementary high-dimensional features through an automatic-learning neural network, and this enables more comprehensive mining of image information. To date, there are six radiomics studies conducted based on deep learning structure in RC. More novel neural network structures are warranted in this field.

RADIOMICS APPLICATIONS IN RC

We searched PubMed (December 17, 2020) for 83 radiomics studies on RC using the terms ("rectal cancer" AND "radiomics"), and identified 78 clinical target-oriented published works. The volume of radiomics-based articles on RC published in medical journals since 2018 has witnessed a steep rise, indicating a trend of growing interest in artificial intelligence-based approaches in the field.

The literature search revealed an exponential growth in RC radiomics studies in the past 3 years. Of the 78 studies, most (61 out of 78) were based on MRI, eight employed CT modality, and nine were based on positron emission tomography/CT or ultrasound images. As of December 2020, most existing studies were performed in a single center with a retrospective cohort, while only seven studies were multi-center. Validation of radiomics models in independent cohorts was performed in all of the studies. However, few studies included external validation cohorts (n = 7). The number of included RC patients ranged widely from 13 to 700. In addition, almost a half of current studies focus on locally advanced RC (LARC) (38 out of 78) or tumor response to nCRT. We suggest that this is mainly because LARC is a major sticking point in the management of RC. Figure 3 shows the distribution of current focus of attention. Here, we discuss recent advances in radiomics applied to CT and MRI for the evaluation of RC.

Response assessment of pre-operative therapy and long-term prognosis prediction in LARC

LARC is of significant concern due to the potential for deterioration. Over the past decade, both disease-free survival and overall survival of LARC have been prolonged, owing to the growing practice of multi-disciplinary treatment and routinization of management, including TME, nCRT, and immunotherapy[3,32]. However, patients tend to present heterogeneous long-term outcomes in clinical practice due to individual responses to preoperative therapy such as short-course preoperative radiotherapy and nCRT[32,33]. Thus, those who are sensitive to chemoradiotherapy may achieve a clinical complete response and employ a watch-and-wait strategy, whereas patients who are resistant to nCRT need more radical measures[34]. Tailoring treatment schemes to a particular patient based on individual probability for achieving a good response is thus essential. The current pre-treatment response prediction approach to nCRT remains indecisive and fails to adequately estimate a patient's response to a specific therapy. Traditional medical imaging methods including CT, quantified MRI, and functional imaging have limitations in response prediction. In contrast, radiomics with high-dimensional feature extraction applied in RC may facilitate the *a priori* evaluation of treatment efficacy and selection of optimal therapy.

Almost 60% of the current research focuses on tumor response assessment to preoperative therapies or prediction of long-term prognosis (n = 49), mainly in LARC (38 out of 49). Most studies to date have evaluated T2-weighted imaging (T2WI) and diffusion weighted MRI. Heterogeneous results have been reported in different prediction models, referring to entropy, energy, and kurtosis. Two well-conducted studies reported that none of the T2WI radiomics features were significant predictors of response[35,36]. SVM, RF, and Naïve Bayesian network based on T2WI yielded

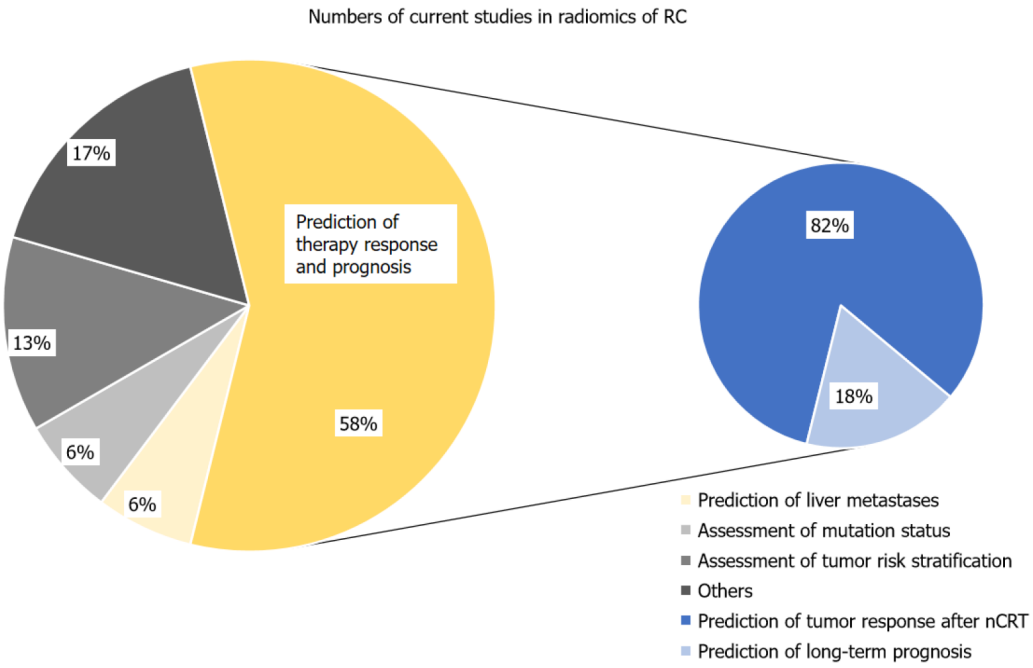


Figure 3 Distribution of the current focus in the industry. Currently 58% of research focuses on tumor response assessment to preoperative neo-adjuvant radiochemotherapy (nCRT) therapies or prediction of the long-term prognosis, of which most studies are about prediction of tumor response after nCRT. RC: Rectal cancer; nCRT: Neo-adjuvant radiochemotherapy.

promising results for complete response (CR) prediction [area under the receiver operating characteristic curve (AUC): 0.71-0.87][35,37,38]. In contrast, apparent diffusion coefficient and intravoxel incoherent motion (IVIM) histogram features did not exhibit predictive value for CR[39]. Similar discrepancies were identified in gray-level co-occurrence matrix (GLCM) dissimilarities for preoperative evaluation of neoadjuvant chemoradiotherapy responses. In this regard, Liu *et al*[39] proposed that GLCM IVIM parameters independently predicted CR in multivariate analysis. Both the random forest model of Yang *et al*[40] and logistic regression model of van Griethuysen *et al*[41] may assist in distinguishing non-insensitive responders to chemoradiotherapy.

Assessment of tumor vascular/perineural invasion (extramural venous invasion/perineural invasion)

Extramural venous invasion (EMVI), which presents in one-third of all patients with RC, is a major factor for higher risk of recurrence and an independent indicator of poorer prognosis[42]. MRI is more suitable to identify the patients with obvious blood vessel invasion beyond the muscularis propria[43]. The verdict of conventional MRI may be affected by surrounding inflammation, edema, and fibrosis caused by nCRT, let alone heterogeneity with image quality, methods, and diagnostic accuracy[44,45]. To obtain more accurate preoperative risk stratification, Yu *et al*[46] constructed and validated several radiomics models and compared them with quantitative models. All radiomics models outperformed quantitative models for predictive performance in identifying EMVI. Notably, the team compared these models with a perfusion parameter-based model from dynamic contrast-enhanced-MRI and demonstrated that the latter had a weaker predictive value.

The positive perineural invasion (PNI) status at resection can independently predict the local recurrence of RC or progression, indicating that the tumor may be of a more aggressive phenotype. According to the latest ESMO guidelines, PNI is a key factor for determining whether patients with stage II RC would potentially benefit from nCRT and postoperative adjuvant chemotherapy[8]. Unlike EMVI, PNI can only be confirmed in post-resection pathological tests, whereas conventional MRI is inoperative for assessing PNI status, as it is unable to visualize peripheral nerves[47]. Therefore, developing a radiomics prediction model to preoperatively identify PNI and to assist in the selection of patients for adjuvant therapy is crucial. Chen *et al*[48] developed a MRI-based radiomics predictive model, which yielded favorable performance for individualized PNI prediction in patients with RC. However, this was a single-center retrospective study based only on T2WI sequences. Future studies

should explore this issue further with heterogeneous and multi-sequence data.

Prediction of synchronous/metachronous liver metastasis

Approximately 15%-20% of patients with RC have liver metastases at the time of diagnosis, which is defined as synchronous liver metastasis (SLM)[49]. At present, only two studies have explored the use of a radiomics nomogram for predicting SLM derived from primary RC lesions[50,51]. Shu *et al*[50] extracted a total of 328 radiomics features from the T2WI images and developed a radiomics model composing T-stage and radiomics signatures. Quantified analysis revealed that the Rad score of patients with primary CRC and liver metastasis was significantly higher than that of patients without liver metastasis. Receiver operating characteristic curves based on all 194 patients with RC were plotted for a radiomics nomogram, and the AUC was 0.932. Liu *et al*[51] subsequently constructed a novel radiomics nomogram and improved the AUC to 0.944, with a sensitivity of 95.83% and specificity of 88.89%, indicating that the radiomics model exhibited good predictive performance for the diagnosis of SLM in patients with RC and may assist physicians in clinical decision-making. The heterogeneity of primary tumors often determines tumor relapse and progression; in contrast, the biological behavior of tumor metastasis and recurrence is closely related to the pathological heterogeneity of the primary tumor. In addition, the radiomics features derived from the primary tumor itself are often more stable in patients with RC. Therefore, radiomics studies examining the prediction of liver metastasis based on primary RC lesions are warranted.

Metachronous liver metastasis (MLM) is defined as the absence of evidence of metastatic disease at initial diagnosis but the presence of liver metastasis after baseline staging and treatment[52,53]. MLM is thought to evolve from occult and micro metastases[54]. Approximately 26.5% of patients with RC develop MLM within 5 years of follow-up[55]. Based on machine-learning algorithms and imaging sequences, noninvasive radiomics models constructed on baseline rectal MRI presented good potential for MLM prediction in patients with RC. The most optimal model employed a logistic regression algorithm, incorporating both T2WI and venous phase radiomics features[56]. To date, reports on MLM prediction based on primary rectal tumors are lacking.

Assessment of genetic mutational status

With the development of targeted therapies such as epidermal growth factor receptor (EGFR)-targeted monoclonal antibody treatment, genetic profiling for mutations is recommended when metastases are diagnosed in patients with RC[8,57]. Previous studies have demonstrated that the mutation statuses of rat sarcoma viral oncogene homolog and v-raf murine sarcoma viral oncogene homolog B1 are critical biomarkers in the prognosis of RC, especially for patients with suspected or proven metastases [58]. Kirsten rat sarcoma (KRAS) mutations, which occur in approximately 27%-43% of patients with RC, have been identified as a critical factor, as they indicate a lack of response to EGFR-targeted therapy[59]. Pathologic tests are the current gold standard of genotyping diagnosis in clinical practice, although results can only be obtained after invasive procedures and may not always be available or reliable. Thus, personalized treatment strategies are warranted to develop a non-invasive and more feasible, timely, and cost-effective surrogate biomarker to evaluate mutation status. With the advent of artificial intelligence (AI) approaches, non-invasive prediction of genetic status and efficacy of CRT using radiomics analysis has become a highlight in the field.

We identified a limited number of studies that used modality-based radiomics models to predict KRAS mutation status or microsatellite instability in RC[60], or to evaluate the relative diagnostic potential of radiomics features for predicting mutational status[59,61-64]. Relationships between radiomics features extracted from CT or multiparametric MRI and KRAS mutations have been confirmed in several studies. Meng *et al*[60] and Xu *et al*[61] reported that radiomics features based on MRI predicted KRAS mutations. Oh *et al*[62] reported that MR-based texture analysis differentiated KRAS mutation status with an accuracy of 81.7%. Furthermore, Cui *et al*[63] used a two-center cohort to predict KRAS mutations in RC and observed that the model obtained with the SVM classifier exhibited the best predictive value. Notably, most of these MRI-derived radiomics models demonstrated moderate predictive ability for KRAS mutations. Further, they were underscored by small sample sizes and lack of independent/external validation. As such, further research is warranted.

FUTURE CHALLENGES AND OPPORTUNITIES

Extant literature has highlighted the potential of radiomics analysis for prediction of nCRT response and prognosis, tumor risk stratification, and evaluation of gene mutation status. Nevertheless, some limitations of radiomics applied for RC should be acknowledged. Current issues arise due to the reliance of results on high-quality data sets, which may bias comparisons of efficacy among radiomics studies due to differences in scanning parameters, feature extraction, software, and vendors/modalities. Hence, there is an urgent need to establish unified data acquisition and processing standards. In addition, radiomics is sensitive to accurate ROI segmentation in the acquisition process. Currently, the process is predominantly conducted manually by radiologists or physicians. Manual segmentation is laborious, and the results may be affected by the observer's subjectivity. The use of semi-automatic or fully automatic methods for segmentation may resolve these issues and should be pursued in future studies. In this regard, accurate and automatic labeling procedures should be developed and promoted to address current technical limitations, and there is still a long way to go. In addition, most existing radiomics studies are retrospective in nature and lack independent external validation across races or populations from various geographical sources, which may limit the reliability and applicability of results. Therefore, further multi-center and prospective studies with standardized acquisition, segmentation, and imaging postprocessing are required to ensure the generalizability and validation of radiomics findings. With regard to mechanistic investigations of biological substrates and their relationships with pathological underpinnings, AI algorithms with improved accuracy and interpretability will facilitate broader translation and clinical adoption. Moreover, radiogenomics for RC is burgeoning, which will bridge the gap between AI-aided prognostics and precision medicine.

As we have discussed above, radiomics models based on deep learning have proven superior. Nevertheless, several issues remain to be optimized; although more parameters exist in the whole connection layer, the gradient of the input end can easily dissipate during network training. A possible solution is to reduce the complexity of the model by reducing network parameters and optimizing the gradient flow, such as VGg, concept, residual network, and dense net.

CONCLUSION

Radiomics is an emerging quantitative technique that has witnessed exponential growth in application of RC management. In this review, we discuss the current utility of radiomics in RC research and describe its potential applications for precision diagnostics and cancer treatment. Thus far, radiomics has shown promise in the diagnosis, treatment evaluation, and prediction of prognosis in RC. Nevertheless, further multi-center and prospective validation studies are required to validate its clinical utility, especially in prognosis-related targets. The purpose of this review is to help radiologists, endoscopists, and oncologists better understand and harness radiomics for tailoring personalized treatments and to encourage their collaboration with AI scientists to promote the translation of research into clinical practice.

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MINIREVIEWS

Advances in paediatric nonalcoholic fatty liver disease: Role of lipidomics

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Abstract

Due its close relationship with obesity, nonalcoholic fatty liver disease (NAFLD) has become a major worldwide health issue even in childhood. The most accepted pathophysiological hypothesis is represented by the "multiple hits" theory, in which both hepatic intracellular lipid accumulation and insulin resistance mainly contribute to liver injury through several factors. Among these, lipotoxicity has gained particular attention. In this view, the pathogenic role of different lipid classes in NAFLD (*e.g.*, sphingolipids, fatty acids, ceramides, *etc.*) has been highlighted in recent lipidomics studies. Although there is some contrast between plasma and liver findings, lipidomic profile in the NAFLD context provides novel insights by expanding knowledge in the intricate field of NAFLD pathophysiology as well as by suggesting innovative therapeutic approaches in order to improve both NAFLD prevention and treatment strategies. Selective changes of distinct lipid species might be an attractive therapeutic target for treating NAFLD. Herein the most recent evidence in this attractive field has been summarized to provide a comprehensive overview of the lipidomic scenario in paediatric NAFLD.

Key Words: Fatty; Liver; Lipidomics; Children; Nonalcoholic fatty liver disease

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Core Tip: Insightful data from lipidomics studies have recently expanded knowledge about nonalcoholic fatty liver disease pathophysiology. In fact, different lipids have been found to exert specific pathogenic roles in liver injury through several pathways

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(in particular by impairing insulin signalling). Given the cardiometabolic burden of nonalcoholic fatty liver disease even in childhood, lipidomics findings might improve strategies for nonalcoholic fatty liver disease treatment by providing novel therapeutic targets.

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INTRODUCTION

An increasing prevalence of nonalcoholic fatty liver disease (NAFLD) up to 25% has been found in adults, but alarming paediatric data have also been reported[1-3]. Due to the obesity epidemic, NAFLD has become the most prevalent liver chronic disease in childhood, affecting 3%-10% of the general paediatric population and up to 50% of obese children with a relevant cardiometabolic burden[2,4]. It includes different degree of hepatic steatosis ranging from simple fat accumulation to steatohepatitis and fibrosis, but its pathogenesis remains to be fully elucidated[1].

To date, "multiple hits" have been recognized in the NAFLD pathophysiology, with a pivotal role of the hepatic intracellular lipid accumulation and insulin resistance favouring liver damage through several factors such as lipotoxicity, oxidative stress, inflammation, genetics, gut axis, metabolic, and dietary factors[3,5]. Among these pathogenic factors, lipotoxicity has gained particular remarkable attention[6]. Lipid induced oxidative stress, inflammation, and cell death have been largely studied as major players of this process, and their interplay represent a critical step in both NAFLD development and progression[6-8]. Of note, it must be considered that the chronic inflammation closely related to lipotoxicity represents one of the most important features of several metabolic diseases such as obesity and type 2 diabetes, resulting in a dangerous "vicious circle" with dramatic clinical implications[6]. In fact, lipotoxicity affects a broad range of tissues such as adipose tissue, heart, brain, pancreatic islets, and skeletal muscle with a complex interrelation favouring the development of metabolic syndrome[6].

Although intrahepatic fat accumulation has been widely accepted as the hallmark of NAFLD, overwhelming evidence showed that both quantity and quality of accumulated hepatic lipids play a pathogenic role in NAFLD[7,9-12]. In particular, specific lipid classes such as sphingosine, diacylglycerols, and ceramides act as liver damaging agents through multiple pathways[6,7,9,13,14].

To date, the growing interest in lipidomic studies has provided meaningful data regarding lipid profiles involved in the pathogenesis of liver injury and its modulation as a potential therapeutic target[8,11,12-14]. In view of the clinical relevance of these findings in the NAFLD treatment scenario, evidence is currently available in adults and in children[15-19] (Tables 1 and 2).

We aimed to summarize the most recent findings regarding lipidomic studies in paediatric NAFLD by providing an overview of the different lipid class and their potential therapeutic implications.

LIPIDS IN NAFLD

Triacylglycerols (TAG) are the most representative lipidic class accumulated in the liver of NAFLD patients. Nevertheless, they would seem to be protective against lipotoxicity due to lipid overload. Lipotoxicity is mainly caused by increasing levels of saturated fatty acids (SFAs), free cholesterol, glycerophospholipids, sphingolipids, and deficient levels of phospholipids, u-3 polyunsaturated fatty acids (PUFAs), or PUFA-derived specialized proresolving mediators[20,21]. Monounsaturated fatty acids (MUFAs), lysophosphatidylcholine (LPC), and ceramide are also increased while phosphatidylcholine (PC) is reduced in nonalcoholic steatohepatitis (NASH)[22,23].

Table 1 Comparison between adult and paediatric lipidomic findings in nonalcoholic fatty liver disease

Lipid class	Changes in adult NAFLD patients	Changes in paediatric NAFLD patients
SFAs	Increased in liver[31] and in plasma[24]	Increased in liver and in plasma[32]
MUFAs	Increased in liver[35,36] and in plasma[25]	Increased in liver and in plasma[32]
PUFAs	Increased total PUFAs in liver[31] and n-6 LCPUFA in liver phospholipids[35]. Decreased LCPUFA of the n-6 and n-3 series in liver TAG, n-3 LCPUFA in phospholipids, total PUFA[35,36], n-3 PUFA[35], n-6 PUFA[32]	Increased in liver and in plasma[32]
PUFAs derived	Increased 5-HETE, 8-HETE, 11-HETE, and 15-HETE in NAFLD and NASH patients[27]. Increased 11,12-diHETrE, dhk PGD2, 20-COOH AA in NASH patients[26]	Increased EDPs, EEQs, EETs with progression of steatosis; reduced with progression of fibrosis[32]
TAG	Increased in liver[31] and in plasma[25]	Increased (TG[O]); TG (O-52:0), TG (O-52:1), TG (O-52:2), TG (O-52:3), TG (O-54:1), TG (O-54:2), TG (O-56:1) and TG (O-56:2) in serum[19]
DAG	Increased in liver[31] and in plasma[25]	No available data
FC	Increased in liver[31]	No available data
PC	Reduced in the liver[31], conflicting data for changes in serum [28,29]	Reduced serum alkyl/alkenyl-phosphatidylcholine (PC[O]) levels[19]
LPC	No statistically significant changes in plasma and serum[28, 29]	No available data
PE	Decreased in liver[22] and increased in serum of NASH patients[28]	Increased PE in serum[19]
LPE	Decreased in serum of patients with NAFLD and NASH[28]	Increased LPE (20:3) and LPE (22:5); decreased [LPE(O)]) in serum[19]
PS	Reduced in liver[22], increased in plasma[29]	No available data
PI	Reduced in liver[22], increased in plasma[29]	No available data
PL	No change in liver[56]; decreased in plasma of NASH patients [25,57]	No available data
SM	Conflicting results in NAFLD and NASH patients[22,28,29,31, 37,51]	Increased SM (d39:0), SM (d41:0) in serum[19]
CE	Increased in liver and in plasma[51,64,65]	Increased in serum[20]

20-COOH AA: 20-carboxy arachidonic acid; CE: Ceramides; DAG: Diacylglycerols; dhk PGD2: 13,14-dihydro-15-keto prostaglandin D2; diHETrE: Dihydroxy-eicosatrienoic acid; EDP: Epoxyeicosapentaenoic acid; EET: Epoxyeicosatrienoic acid; EEQ: Epoxyeicosatetraenoic acid; FC: Free cholesterol; HETE: Hydroxyeicosatetraenoic acid; LCPUFA: Long chain polyunsaturated fatty acid; LPC: Lysophosphatidylocholine; LPE: Lysophosphatidylethanolamine; LPE(O): Alkyl/alkenyl-lysophosphatidylethanolamine; MUFAs: Monounsaturated fatty acids; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; PI: Phosphatidylinositol; PL: Plasmalogens; PS: Phosphatidylserine; PUFAs: Polyunsaturated fatty acids; SFAs: Saturated fatty acids; SM: Sphingomyelin; TAG: Triacylglycerols; TG(O): Alkyl-diacylglycerols.

Of interest, lipidomic studies conducted in both plasma and serum of NAFLD patients reported overlapping results with those found in the liver. In addition, increased levels of total SFA in phospholipids[24], metabolites of lipoxygenase, 5-hydroxyeicosatetraenoic acid (HETE), 8-HETE[25], 15-HETE, 5,6 dihydroxy- eicosatrienoic acid[26], palmitoleic acid in cholesteryl ester[27], PC and sphingomyelin[28], phosphatidylserine, and phosphatidylinositol[29] were found in plasma and serum of NAFLD patients. On the other hand, decreased levels of eicosanoic acid (C20: 0), cis-11-octadecenoic acid (C18: 1n-7), docosahexaenoic acid in phospholipids[24], 12,13-dihydroxy-9-octadecenoic acid[26], and lysophospatidylethanolamine[28] have been described.

Fatty acids

The increased share of free fatty acids reaching the liver in NAFLD has been related to three main mechanisms: lipolysis of adipose tissue, *de novo* lipogenesis, and diet[30].

Both hepatic[31] and plasma[24] findings from adults and children[32] with NAFLD showed an increased content of SFAs. Of concern, SFAs seem to be one of the major players involved in lipotoxicity. In fact, evidence linked inhibition (genetic or pharmacological) of the enzyme converting SFAs to MUFAs, namely stearoyl-CoA desaturase-

Table 2 Main findings of lipidomic studies in paediatric nonalcoholic fatty liver disease

Ref.	Study design and methods	Population (*n*)	Main findings
Wasilewska *et al*[20], 2018	Prospective study	80 children at median age 12 (7-17 yr)	Higher total serum CE concentration in NAFLD patients, compared to the controls and of certain CEs (C14:0, C16:0, C16:1, C18:0, C18:1, C22:0, C24:0). Total CE concentration was positively correlated with HOMA-IR and insulin levels
Draijer *et al* [8], 2020	Case-control study	21 children with obesity and steatosis and 21 with only obesity. Mean age of NAFLD patients: 14.8 yr; mean age of non-NAFLD patients 14.7 yr	Statistically significant alterations in 5 major lipid classes [TG(O), PE, PE(O), LPE(O), PC(O)] and 12 individual lipid species
Kalveram *et al* [32], 2021	Prospective study	40 children with biopsy-proven NAFLD. Mean age 14.2 ± 2.3 yr	Hepatic epoxyeicosanoids levels increased with higher degrees of steatosis. CYP epoxygenase activity increased, protein level, and activity of sEH decreased. In contrast, hepatic epoxyeicosanoids decreased with higher stages of fibrosis, with a decrease of CYP epoxygenase activity and protein expression

CE: Ceramides; CYP: Cytochrome P450; HOMA-IR: Homeostasis model assessment; NAFLD: Nonalcoholic fatty liver disease; LPE(O): Alkyl/alkenyl-lysophosphatidylethanolamine; PC(O): Alkyl/alkenyl-phosphatidylcholine; PE: Phosphatidylethanolamine; PE(O): Alkyl/alkenyl-phosphatidylethanolamine; sEH: Soluble epoxide hydrolase; TG(O): Alkyl-diacylglycerols.

1, to different processes such as hepatocyte apoptosis, lipotoxicity, and development of steatohepatitis[33]. Consequently, balancing between MUFAs and SFAs might represent a central player in the progression from isolated hepatic steatosis to progressive steatohepatitis and fibrosis[34].

MUFAs were also found to be increased in liver and plasma of both adult and paediatric patients with NAFLD[25,32,35-37]. This lipid class has been considered as less lipotoxic than SFAs because channelling free fatty acids into MUFAs allow their incorporation into triglycerides and storage in lipid droplets[34].

The long chain PUFAs such as eicosapentaenoic, docosahexanoic, and arachidonic acids were decreased in liver and plasma of patients with NAFLD[29,31,35,36]. This reduction could be due to impairments in dietary intake or the biosynthesis process. A pivotal pathogenic role in NAFLD progression (from simple steatosis to NASH) has been attributed to decreased activity of fatty acid desaturase 1, an enzyme involved in the PUFAs metabolism[6].

Through the activity of hepatic cytochrome P450 enzymes, PUFAs derived from monoepoxides are collectively termed epoxyeicosanoids[38-40]. Those deriving from arachidonic acid (epoxyeicosatrienoic acid), eicosapentaenoic acid (epoxyeicosatetraenoic acid), and docosahexaenoic acid (epoxyeicosapentaenoic acid) have anti-inflammatory, antisteatotic, and antifibrotic properties[41]. In a recent paediatric study [32], 40 youths with biopsy-proven NAFLD underwent lipidomic evaluations by analysing liver tissue and blood samples. Upregulated hepatic epoxyeicosatrienoic acid, epoxyeicosatetraenoic acid, and epoxyeicosapentaenoic acid levels were found in children with steatosis. This might be due to reduced activity and protein expression of soluble epoxide hydrolase, metabolizing epoxyeicosanoids to vicinal diols. On the contrary, at the stage of fibrosis the aforementioned epoxyeicosanoids were found to be decreased in liver and plasma because of a potential reduction of cytochrome P450 epoxygenase expression. Therefore, the cytochrome P450 epoxygenase/soluble epoxide hydrolase pathway seem to represent a potential innovative pharmacologic target for NAFLD treatment.

Proinflammatory molecules are also derived from PUFAs. Puri *et al*[25] found increased plasma levels of arachidonic acid (5-HETE, 8-HETE, 11-HETE, and 15-HETE) lipoxygenase metabolites in NAFLD and NASH patients compared to lean normal controls. Moreover, plasma arachidonic acid-derived metabolites 11,12-dihydroxy-eicosatrienoic acid, 13,14-dihydro-15-keto prostaglandin D2, and 20-carboxy arachidonic acid levels were found to be significantly increased by Loomba *et al*[26] in subjects with NASH than those with NAFLD.

With respect to the wide cardiometabolic burden of NAFLD, changes in FA metabolism have also been linked to its related comorbidities including obesity, diabetes, and cardiovascular risk[20,28,29].

Neutral lipids

The hallmark of NAFLD is the accumulation of lipid droplets in the hepatocytes containing TAG[7]. TAGs were found to be increased in both plasma and liver of

patients with NAFLD compared to healthy controls[25,31]. They represent a less toxic form of storing lipids. The inhibition of diacylglycerol acyltransferase 2, the enzyme catalysing the final step in the assembly of TAG, reduced steatosis but at the same time increased hepatic free fatty acids, lipid peroxidation, oxidative stress, necroinflammation, and fibrosis[42]. In mice defective for perilipin-5, a protein binding lipid droplet and regulating TAG storage, the reduction in the size of lipid droplets caused increased lipolysis and lipotoxicity[43].

Diacylglycerols (DAG) were also increased in plasma and liver of patients with NAFLD[25,31], and the ratio of TAG/diacylglycerols seemed to increase in the evolution from NAFLD to NASH[31].

In a paediatric study, Draijer *et al*[8] performed lipidomic analyses in plasma samples of 21 children with obesity and proton magnetic resonance spectroscopy-detected hepatic steatosis compared to the lipidome of 21 samples of nonsteatotic subjects with obesity. The authors found an overall significant increase in NAFLD patients of serum alkyl-diacylglycerols [TG(O)], in particular 8 TG(O) species (TG(O-52:0), (TG(O-52:1) TG(O-52:2), TG(O-54:1), TG(O-54:2), TG(O-52:3), TG(O-56:1) and TG(O-56:2)].

Finally, it should also be noted that the amount of hepatic free cholesterol increases with the progression to NASH, without an increase in cholesterol esters[31]. It is considered a cytotoxic lipid disrupting membrane integrity and inducing oxidative stress, mitochondrial dysfunction, and apoptosis[44].

Glycerophospholipids

Glycerophospholipids represent a significant lipidic fraction of the cell membrane. Reduced hepatic PC levels were observed in both NAFLD and NASH subjects[22,31]. However, conflicting data about alterations in serum were reported[6]. Low hepatic levels of PC influenced circulating very low-density lipoproteins, which are therefore reduced with consequent hepatic accumulation of TAG[45,46]. Low levels also increased de novo lipogenesis and formation of lipid droplets in hepatocytes by activation of sterol regulatory element-binding protein 1[47].

Liver phosphatidylethanolamine (PE) content was found to be decreased among subjects with NASH[22], while serum PE increased[28]. The enzyme phosphatidylethanolamine n-methyltransferase catalyses the reaction converting PE to PC. Studies reported that a loss-of-function polymorphism in the phosphatidylethanolamine n-methyltransferase gene predisposed to NAFLD susceptibility[48]. Lower hepatic PC/PE ratio was also reported in NAFLD individuals. Interestingly, a reduced PC/PE ratio in red blood cell membranes has been found to enhance predisposition to NAFLD[49]. As a consequence, loss of membrane integrity and higher permeability to proinflammatory factors were observed[50].

Paediatric data reported significantly increased PE serum levels and reduced specific etherphospholipid classes such as alkyl/alkenyl-phosphatidylethanolamine, alkyl/alkenyl-lysophosphatidylethanolamine [LPE(O)], and alkyl/alkenyl-phosphatidylcholine in subjects with NAFLD. When looking at individual lipid species, two LPE species such as LPE (20:3) and LPE (22:5) were found to be increased[19].

Phosphatidylserine and phosphatidylinositol were decreased in the liver[22] but increased in the plasma[29] of NAFLD patients. However, these results are conflicting in other studies[25,28,51].

LPC was increased in the liver of NASH patients[6], while no statistically significant changes in plasma and serum of LPC content were reported in patients with NAFLD or NASH[28,29]. LPC derived from PC by the action of lipoprotein associated phospholipase A2 at the intracellular level, whereas in the extracellular milieu by lecithincholesterol acyltransferase activity. In patients with NAFLD, phospholipase A2 levels were found to be decreased, while those of lecithincholesterol acyltransferase increased[52,53]. LPC downregulates genes involved in fatty acid oxidation, upregulates genes involved in cholesterol biosynthesis, and promotes apoptosis of hepatocytes[54]. Inhibitors of phospholipase A2 decreased palmitate-induced lipotoxicity and cell apoptosis[54,55].

In the liver of NAFLD patients no change in plasmalogen content was reported[56], while this class was decreased in the plasma of NASH patients[25,57]. Animal data demonstrated that a specific mechanism (involving peroxisome proliferator-activated receptor-alpha) sustained by endogenous hepatic plasmalogens may prevent liver steatosis and NASH[58].

Circulating plasmalogen levels, particularly 16:0 and 18:1, were found to be reduced in NAFLD individuals with the GG-genotype of patatin-like phospholipase domain-containing 3 (*PNPLA3*) compared to those with the C or CG allele[28]. The *PNPLA3* gene is highly expressed in hepatic stellate cells of the liver and adipose tissue and

encodes adiponutrin, a protein exerting both lipase and acyltransferase activity[59]. Adiponutrin variant p.I148M [rs738409 (G)] enhanced PUFA content of TAGs and diacylglycerols and negatively affected both PC synthesis and lipid droplet hydrolysis [60].

An elegant paediatric study examining NAFLD genetic factors demonstrated that *PNPLA3* rs738409 (G) represented the strongest determinant of the presence of NAFLD as compared to healthy controls and conferred the highest risk of severity of steatosis. Interestingly, a specific steatosis pattern (including an increased percentage of portal inflammation) was reported in homozygous *PNPLA3* rs738409 (G) patients [61].

In addition to NAFLD, a significant association of these compounds has been found in a larger cardiometabolic-related context such as obesity and cardiovascular disease [51,57].

Sphingolipids

Sphingolipids are structural components of cellular membranes and signalling molecules in mammalian cells. Conflicting results were found about sphingomyelin (SM) trends in NAFLD and NASH patients[22,28,29,37,51]. Barr *et al*[62] found an increase in serum levels of certain sphingomyelin species, such as SM (36:3), (d18:2/16:0), (d18:2/14:0), (d18:1/18:0), (d18:1/16:0), (d18:1/12:0), and (d18:0/16:0) in NAFLD individuals compared to controls. Instead, reduced circulating levels of SM (d18:1/24:1), SM (d18:1/16:0), SM (d18:1/22:0), SM (d18:1/24:0), SM (d18:1/18:0), SM (d18:1/20:0), SM (d18:1/23:0), SM (d18:0/16:0), and SM (d18:0/20:4) were observed by Zhou *et al*[63] in NASH adult subjects compared to controls. Moreover, increased serum levels of two SM species such as SM(d39:0) and SM (d41:0) were found in the serum of NAFLD paediatric patients[19].

Higher ceramide levels were found in plasma and liver biopsies of NAFLD subjects [51,64,65]. These lipids decreased insulin sensitivity in skeletal muscle and hepatocytes [66] and enhanced several unfavourable biological processes such as oxidative stress, mitochondrial dysfunction, and cell apoptosis[66,67]. Moreover, they seem to regulate the synthesis of high-density lipoproteins. Animal data reported that myriocin, acting through ceramide biosynthesis inhibition–promoted insulin receptor and steatosis and enhanced apolipoprotein AI production rate, resulting in an increased high-density lipoprotein production rate[68].

In a prospective study[20] including 80 obese children, total ceramide concentration was significantly increased in the serum of obese and NAFLD patients than in the reference group. In addition, increased levels of distinct fatty acid ceramides, such as myristic, palmitic, palmitoleic, stearic, oleic, behenic, and lignoceric were observed in children with NAFLD compared to controls. Furthermore, a significant positive association of total ceramide levels with homeostasis model assessment and insulin levels was reported[20].

Taken together, these findings might pave the way for a wider risk assessment for these patients, as suggested by paediatric evidence indicating a significant association of distinct sphingolipids with NAFLD and with its cardiometabolic burden including obesity, cardiovascular disease, and metabolic derangements[20,69-71].

CONCLUSION

Lipidomic studies have added novelty by allowing an accurate characterization of lipidomic profile of both plasma and liver tissues in NAFLD[7,12,17]. Besides experimental data providing additional insights about the pathophysiology of NAFLD and its progression, there is a growing body of evidence from human studies[8,14,20]. In particular, a clear effect for specific ceramides in impairing insulin signalling pathways has been found[10,13,15].

Interestingly, different lipid classes have been demonstrated to exert pathogenic distinct roles in NAFLD and in other metabolic diseases such as obesity, metabolic syndrome, and type 2 diabetes[14,15]. Thus, manipulation of the expression of certain lipids (*e.g.*, selective lowering of specific ceramides) might represent a novel target for both prevention and treatment of these diseases. In fact, this attractive therapeutic approach might pave the way for novel strategies to counteract the increasing NAFLD-related cardiometabolic burden even in childhood.

Further research is needed to validate these findings and to provide a more comprehensive assessment of the exact pathogenic role of specific lipids in the NAFLD context.

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MINIREVIEWS

Autoimmune pancreatitis and pancreatic cancer: Epidemiological aspects and immunological considerations

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Abstract

Ordinary chronic pancreatitis is a well-known risk factor for pancreatic cancer, whereas such an association with autoimmune pancreatitis (AIP) is widely debated. Due to the rarity of the latter disorder, there are few specific clinical and epidemiological studies investigating the relation between AIP and pancreatic cancer, which do not seem to support it. However, these studies are affected by several limitations and, therefore, a link between AIP (and, specifically, type 1 AIP) and pancreatic cancer cannot be ruled out definitively on this basis. Moreover, several immunopathological aspects of type 1 AIP and, in general, immunoglobulin G4-related disease can create an immunological context that may impair the tumoral immunosurveillance and promote the pancreatic carcinogenesis and its progression. In detail, Th2 immunological dominance, type 2 macrophage polarization and basophil infiltration observed in type 1 AIP, may play a permissive role in creating a favorable immunological environment for pancreatic carcinogenesis, in addition to the immunosuppressive therapies that can be used in these patients.

Key Words: Autoimmune pancreatitis; Chronic pancreatitis; Pancreatic cancer; Immunoglobulin G4-related disease; Epidemiology; Immunology; Basophils; Macrophages; Th2 cells; Systemic lupus erythematosus

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Core Tip: This mini-review discusses the debated issue of autoimmune pancreatitis (type 1) as a potential risk factor for pancreatic cancer. After summarizing the few

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available (low-quality) epidemiological evidence that does not clearly support this role, the immunopathological characteristics of type 1 autoimmune pancreatitis (including Th2 immunological dominance, type 2 macrophage polarization and basophil infiltration) are discussed as potential factors that may actually create a tolerogenic immunological environment favorable to pancreatic carcinogenesis and/or tumor progression.

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INTRODUCTION

Autoimmune pancreatitis: Definition, pathology, and epidemiology

Chronic pancreatitis (CP) is a persistent inflammatory disease of the exocrine pancreas leading to progressive fibrotic tissue damage. CP prevalence varies between 13.5-98.7 cases per 100000 people with an incidence of around 4-5 new cases per 100000 people every year. Most cases of CP (also defined as "ordinary" or "generic") are triggered by the repeated and/or persistent activation of intrapancreatic digestive enzymes, due to the variable combination of some environmental factors (*e.g.*, excessive alcohol consumption, regular tobacco use, hypertriglyceridemia, *etc.*) and/or genetic factors (*e.g.*, Chymotrypsin C mutations)[1].

Autoimmune Pancreatitis (AIP), which represents < 5%-10% of pancreatitis cases and has an estimated prevalence of approximately 1-2/100000 people, is much rarer[1,2]. However, the real incidence of AIP is currently unknown: indeed, it is diagnosed in around 2% of patients who have undergone pancreas surgical resection for presumed pancreatic cancer and, therefore, this condition may be underdiagnosed[3].

The term AIP was originally introduced by Yoshida *et al*[4] in 1995 to describe a patient diagnosed with pancreatitis who "had hyperglobulinemia, was autoantibody-positive, and responded to steroid therapy". Currently, AIP diagnosis must be supported by well-defined and specific clinical, radiological, serological and histopathological criteria[3]. Importantly, based on histopathological aspects (according to the Honolulu classification), AIP is classified into two main subtypes that are mainly distinguished by the absence (type 1 AIP) or presence (type 2 AIP) of one peculiar finding, called "granulocytic epithelial lesion"(GEL), consisting of the neutrophilic infiltration of medium/small-sized ducts and often acini[5]. Thus, type 1 AIP is characterized by a substantial lymphoplasmacytic infiltration, where eosinophils may be relatively frequent and neutrophils are rare. Indeed, it was originally described as lymphoplasmacytic sclerosing pancreatitis[5,6]. Moreover, it is much more frequent than type 2 AIP, which accounts for 10%-20% and no more than 5% of all AIP cases in Western and Eastern countries, respectively. Importantly, type 1 AIP is now accepted as a clinical-pathological aspect of immunoglobulin G4 (IgG4)-related disease (IgG4-RD)[6,7].

IgG4-RD is a multisystem immune-mediated fibroinflammatory condition where several organs can be involved and, most frequently, pancreas, bile ducts, salivary glands, lacrimal glands, kidneys, retroperitoneum, and lungs. Pancreas is involved in at least 45% of IgG4-RD patients as type 1 AIP[8]. Therefore, unlike ordinary CP, type 1 AIP is usually the pancreatic manifestation of a systemic immunological disorder, in which several alterations of the innate and acquired immune system contribute to the lympho-plasmocytic inflammatory infiltration of the pancreas[9].

IgG4-RD pathogenesis is complex and involves both the innate and adaptive immune system; as regards the latter one, Th2 (specifically, follicular Th2 cells, Tfh2) and Treg lymphocytes (and related cytokines) have been clearly and mainly implicated in this pathologic process characterized by altered B cell and plasma cell activation, enhancement of IgG4 class switch recombination and, finally, development of ectopic germinal centers and induction of fibrosis[9,10].

Type 2 AIP pathogenesis is more elusive and, indeed, is also called idiopathic duct-centric pancreatitis. It is not characterized by IgG4 increase and other specific extra-pancreatic manifestations, except for an association with inflammatory bowel diseases

in 20%-30% cases[6,7].

Type 1 and type 2 AIP cannot be certainly differentiated by radiological imaging and share similar clinical presentations. However, the diagnostic differentiation is very important since the prognosis is quite different. Indeed, type 1 AIP is usually more aggressive, showing a much higher rate of relapse after steroid therapy, and is characterized by synchronous or metachronous extra-pancreatic organs involvement, as previously mentioned[11].

Both AIP types are almost exclusively adulthood diseases with a median age at diagnosis between 40-50 years, even though patients with type 1 AIP are 10-15 years older on average. AIP has been rarely described in the pediatric population, where the few reported cases were consistent with type 2 AIP in most cases[7,12].

PANCREATIC CANCER AND AIP: EPIDEMIOLOGICAL ASPECTS

Pancreatic cancer is represented by adenocarcinoma in > 85% of cases. Nowadays the overall incidence of pancreatic cancer is estimated to be around 11.0 per 100,000 people per year in the United States, where it accounts for 3% of all diagnosed malignancies. These numbers make it be the twelfth and eleventh most common cancer in men and women, respectively. Pancreatic cancer remains a highly fatal malignancy: Worldwide, it is the seventh leading cause of cancer death in both genders, with a 5-year survival rate of 10% or less. Like most malignancies, incidence rates for pancreatic cancer are age-related: In general, it is rare before the age of 50 years. The main risk factors for pancreatic cancer are modifiable and include tobacco smoking, obesity, physical inactivity and high-calorie/fat diets; these lifestyle-related risk factors clearly contributed to the increase of the disease incidence in the last three decades, especially in developed countries, where diagnostic improvements and increased life expectancy have played a role as well[12,13].

CP (and, in detail, ordinary CP) was shown to be a clear risk factor for pancreatic cancer. In 1993 Lowenfels *et al*[14] published the results of a large multicenter and international cohort study including 2,015 patients with CP, who showed an increased risk of pancreatic cancer, independent from gender, country and type of pancreatitis. It was estimated that approximately 5% of CP patients receive a diagnosis of pancreatic cancer within 2 decades after that diagnosis[12,14].

The potential association between pancreatic cancer and AIP is currently debated. Even though AIP is a rare form of CP, this issue is raised especially in patients affected with type 1 AIP that, compared to type 2 AIP, is more common and characterized by high percentages of disease relapse after steroid therapy[15,16].

Moreover, as summarized and discussed below, all reported cases of AIP-related pancreatic cancer were described in patients affected with type 1 AIP; actually, some concerns were raised in terms of potential association with malignancies in general. Most studies on IgG4-RD and/or type 1 AIP are from Japan: Yamamoto *et al*[17] and Shiokawa *et al*[18] first reported an overall increase of malignancy incidence in their patients, even though they actually described no cases of pancreatic cancer.

A larger multi-centric and international study by Hart *et al*[15] (including 1,064 patients with AIP and, exactly, 978 with type 1 and 86 with type 2) reported 5 diagnoses of pancreatic cancer in type 1 AIP patients (no one affected with type 2 AIP developed this complication). However, as discussed by the authors themselves, even though they found a small number of pancreatic cancers (compared to the total cumulative frequency of malignancies, n = 57), the limited follow-up and lack of a control population may have limited the clinical significance of their analysis. Moreover, this study (and also another one authored by the same research group) were not focused on pancreatic cancer[15,19], as well as the following studies. Hirano *et al*[20] described their case series of 113 IgG4-RD patients (type 1 AIP: n = 95) and reported 2 patients who developed pancreatic cancer; in general, their conclusion was that the cumulative incidence of any kind of malignancies in IgG4-RD patients was similar to that observed in the general population. Shimizu *et al*[21] also made the same conclusion in their 84 type 1 AIP patients; in detail, among 9 patients diagnosed with cancer, only one developed it at the pancreas. Buijs *et al*[22] analyzed a Dutch case series of 107 patients affected with AIP (type 1: 90%, type 2: 10%), and reported no apparent difference in malignancy incidence compared to an ethnic-, age-, and sex-matched reference population (in detail, no patients developed pancreatic cancer).

However, although several clinical studies tried to address this issue, the prevalence and risk assessment of pancreatic cancer in the pathological setting of AIP have been greatly hampered by the relatively small number of participants, variable and/or

relatively short follow-up period, retrospective study design and the lack of appropriate control groups in most of these studies[23].

Therefore, some authors proposed that patients affected with AIP should be regularly monitored to reveal any potential cancerous changes and/or malignancy onset, anyway[24]. For instance, unlike the aforementioned studies, Huggett *et al*[16] reported a statistically significant odd ratio increase (odds ratio = 2.2) for all malignancy risk in type 1 AIP patients: 13 malignancy cases (out of 115 type 1 AIP and/or IgG4-RD and, 106 patients with pancreatic disease) were diagnosed, and all those tumors arose in systemic IgG4-RD patients, who represented only 56% of the whole cohort. However, only one case was affected with pancreatic adenocarcinoma.

Additional and interesting research articles, which were more focused on pancreatic complications and carcinogenesis in AIP patients, were published in the last few years. A clinical research by Gupta *et al*[25] investigated the pancreatic carcinogenesis in patients affected with AIP. These authors reviewed a case series of 84 AIP patients, and they compared the prevalence of pancreatic intraepithelial neoplasia (PanIN) in 28 cases of AIP and 30 cases of CP not otherwise specified. Overall, 82% AIP patients showed PanIN with variable histologic grade, which resulted to be more frequent than in non-autoimmune CP (63%), though such a difference was not statistically significant. Therefore, the prevalence of PanIN in AIP resulted to be comparable to ordinary CP; moreover, in the same article the authors also described their clinical experience with 84 AIP patients, and the only 2 cases of pancreatic cancer were diagnosed in the context of type 1 AIP, which was diagnosed 6 and 10 years before the detection of the malignancy, respectively. Therefore, this study raised some specific concerns as regards the risk of pancreatic malignancy in type 1 AIP patients[25]. Similarly, Ikeura *et al*[26] diagnosed 3 cases of pancreatic cancer (4.8%) in their 63 type 1 AIP patients, which was similar to what observed in the comparison group, consisting of 41 patients affected with ordinary (alcoholic and hereditary) CP, characterized by only one patient (2.1%) with a diagnosis of pancreatic malignancy.

Starting from a different perspective, Ngwa *et al*[27] described 548 patients diagnosed with pancreatic cancer and 99 different patients affected with type 1 AIP. In this study, whose main aim was to compare the IgG4 serum profiles between those two groups of patients (rather than investigating an epidemiological and/or causal link), the authors suggested no relationship between AIP and pancreatic cancer; however, a minority of histological specimens (only 30 pancreatic tissue specimens from patients with pancreatic cancer and 6 patients with "high-risk features" AIP) was reviewed. Indeed, a recent retrospective study by Xiang *et al*[28], including 74 patients with type 1 AIP, revived the debate by reporting that 5 of them (6.7%) were concomitantly diagnosed with a pancreatic tumor (pancreatic ductal adenocarcinoma, $n = 3$; solitary extramedullary plasmacytoma, $n = 1$; cholangiocarcinoma, $n = 1$). Moreover, an interesting observation was reported by Hedayat *et al*[29], who re-examined 21 pancreas specimens of previously diagnoses of intraductal papillary-mucinous neoplasm, which is a cystic and usually benign neoplasm, but with the potential for progression to pancreatic cancer. Interestingly, 4 of them (19%) showed infiltrates of IgG4-positive plasma cells, consistent with a "peri-tumoral" type 1 AIP reaction[29,30].

Conversely, two very recent retrospective studies were not supportive in this sense. Tang *et al*[31] described 17 neoplastic cases in a large cohort of 587 Chinese patients diagnosed with IgG4-RD; among those, 11 were also affected with AIP, but all developed extra-pancreatic malignancies only. The study by Ishikawa *et al*[32], including 123 type 1 AIP patients, identified only 2 patients diagnosed with pancreatic cancer (1.6%) and concluded that "AIP is unlikely to be a precancerous condition of the pancreas", but at the same time they stated that "because of the small number of cases, the characteristic findings of pancreatic cancers that develop in AIP patients are not clear".

Therefore, as summarized by these two final statements, this short and schematic overview of clinical studies provides conflicting evidence and conclusions regarding the association between AIP (and, in detail, type 1) and pancreatic cancer. Table 1 summarizes all the aforementioned clinical studies investigating the association between AIP and pancreatic cancer, by using a chronological order and focusing on the main study features and findings.

Even though one may conclude that most of the available studies do not support this association, there are some clinical and pathological observations coming from studies more focused on pancreatic cancer[25,26,28] which should keep high the attention on this issue before making final conclusions; however, this will require larger, prospective, and longer (in terms of follow-up period) studies. Unfortunately, the clinical research on this topic is undoubtedly hampered by the low prevalence and incidence of AIP, in general and in the landscape of CP, in addition to the challenges of

Table 1 Schematic overview of the clinical studies investigating the association between autoimmune pancreatitis and pancreatic cancer.

Ref.	Study population (disease)	Total patients (*n*)	AIP type 1 (*n*)	Cancer (overall) (*n*)	Pancreatic cancer (*n*)	Median follow-up (yr)	Additional specifications
Yamamoto *et al* [17] (Japan, 2012)	IgG4-RD	106	10	2	0	NA	-
Shiokawa *et al* [18] (Japan, 2013)	AIP	108	104	18	0	3.3	These 18 malignancies were diagnosed in 15 patients.
Hart *et al*[15] (International, 2013)	AIP	1064	978	57	5	NA	No patients with AIP type 2 developed any malignancies.
Gupta *et al*[25] (United States, 2013)	CP	58	11	NA	7 (PanIN)	N/A	Retrospective analysis of patients with CP. There was no statistically significant difference in the frequency of PanIN between ordinary CP and AIP: in the latter group, no difference between type 1 and type 2. In general, the only case of PanIN3 was detected in one AIP type 1 specimen.
Gupta *et al*[25] (United States, 2013)	AIP	84	NA	NA	2	4.1	These 2 cases of pancreatic cancer were diagnosed in type 1 AIP patients.
Hart *et al*[19] (United States, 2014)	AIP	116	116	23	1	3.6	-
Hirano *et al*[20] (Japan, 2014)	IgG4-RD	113	95	14	2	6	2 IgG4-RD patients diagnosed with malignancy (out of 14) were not affected with AIP.
Huggett *et al*[16] (United Kingdom, 2014)	IgG4-RD	115	106	13	3	2.7	Of these 3 cases of pancreatic malignancies, 2 were cholangiocarcinoma cases and 1 was a pancreatic adenocarcinoma.
Ikeura *et al*[26] (Japan, 2014)	AIP	63	63	NA	3	5.2	-
Shimizu *et al*[21] (Japan, 2015)	AIP	84	84	9	1	4.5	-
Buijs *et al*[22] (The Netherlands, 2015)	AIP	107	96	8	0	6.25	-
Ngwa *et al*[27] (United States, 2015)	AIP	99	99	NA	0	NA	The aim of this study was to evaluate the clinical significance of elevated sgG4 levels in patients with AIP and pancreatic cancer and potential prognostic implications of those in patients with pancreatic cancer.
Xiang *et al*[28] (China, 2019)	AIP	74	74	NA	4	NA	3 patients were diagnosed with pancreatic ductal adenocarcinoma and 1 with cholangiocarcinoma. There was also 1 case of solitary extramedullary plasmacytoma.
Tang *et al*[31] (China, 2020)	IgG4-RD	587	NA	17	0	5.1	-
Ishikawa *et al*[32] (Japan, 2020)	AIP	123	123	NA	2	4.6	-

AIP: Autoimmune pancreatitis; CP: Chronic pancreatitis; IgG4-RD: Immunoglobulin G 4-related disease; N/A: Not applicable; NA: Not available; PanIN: Pancreatic intra-epithelial neoplasia.

pancreatic diagnostics.

Nonetheless, it is established that chronic inflammatory processes represent a risk factor for pancreatic cancer and, as such, type 1 AIP, especially if relapsing and/or persistent, should be considered in the same way in principle. Moreover, concomitant immunosuppressive therapies (including steroids, rituximab, azathioprine., *etc.*) may represent additional predisposing factors to the cancer development[11]. Finally, some specific immunological aspects and considerations (also related to IgG4-RD pathogenesis) might support these concerns, as discussed in the next sections.

TYPE 1 AIP: GENERAL IMMUNOPATHOGENESIS

IgG4-RD is the clinical expression of a systemic immunological dysregulation leading to chronic inflammation and lymphocyte infiltration in several organs (including pancreas), which might have some implications in terms of pancreatic and extra-pancreatic carcinogenesis[8]. However, as discussed, the available clinical evidence does not support this hypothesis, but the small number of studies, which are also characterized by several and important limitations, likewise do not allow to rule out it either.

As said, type 1 AIP is the prevalent form of AIP and all cases of AIP-related pancreatic malignancies were described in this pathological setting. Therefore, the following immunological considerations on AIP immunologic pathogenesis and its potential role in pancreatic carcinogenesis, will refer to type 1 AIP and/or IgG4-RD specifically.

Even though the pathogenesis of IgG4-RD is complex and relies on both innate and acquired immune system mechanisms, the final result is an immunological environment characterized by a Th2 dominant immune response, which is indeed associated with increased level of serum IgG4 (systemically) and IgG4 switched lymphocytes and plasma cells (in the pancreas and other affected organs). Therefore, IgG4-RD and type 1 AIP are characterized by a cytokine profile whereby interleukin (IL)-4, IL-5, IL-13, IL-10 and transforming growth factor (TGF)-β are overexpressed [33].

Recent studies evidenced the central role that is played by the T follicular helper cells (Tfh cells), which can be found in the extra-nodal ectopic germinal centers and are involved in the generation of long-lasting humoral responses by B cells and plasma cells[9]. Increased levels of Tfh cells and, in detail, Th2-polarized cells (Tfh2 cells), have been described in IgG4-RD patients, in whom those cells correlate with serum IgG4 and IL-4 levels[34]. Moreover, Tfh2 cells, but not Tfh1 or Tfh17 cells, were demonstrated to induce the differentiation of naïve B cells into plasma blasts (with enhanced production of IgG4) and, importantly, the activation of Tfh2 cells resulted to correlate with the disease activity[35].

However, in addition to the probable intrinsic dysregulation of the adaptive immune system, the origin of these Tfh2 expansion and activation (and, more in general, Th2 predominance) must be sought in the innate immune system as well[9]. Through the use of an experimental murine model of AIP (namely, MRL/Mp mice treated with polyinosinic-polycytidylic acid), Arai *et al*[36] described the pancreatic accumulation of plasmocytoid dendritic cells (pDCs) producing IFN-α. They also showed that pDCs from human patients with type 1 AIP had an increased production of IFN-α, which was able to promote the B cells switch toward IgG4 production in a T-cell independent manner, probably by the enhanced production of BAFF (B cell-activating factor belonging to the tumor necrosis factor family). Actually, BAFF production upon Toll-like-receptor (TLR) activation was described in other innate immune cells, including basophils, through the stimulation of TLR2 and TLR4[37]. This finding is particularly interesting because the presence of basophils has been recently described in the pancreatic tissue of most patients with type 1 AIP[38].

Basophils have been demonstrated to be more than simple effector cells in several pathological contexts (including asthma and other allergic diseases) and, in detail, were proposed as one of the main sources of early IL-4, being able to drive and/or support the Th2 polarization of activated CD4+ T cells[39-41]. Overall, these observations may support the fact that basophils are activated in a IgE- (and, thus, B-cell) independent manner and, indeed, TLR-activated basophils can contribute to drive the Th2 response in type 1 AIP[38].

Interestingly, in a recent review article, Watanabe *et al*[9] highlighted several immunological similarities between IgG4-RD and Systemic Lupus Erythematosus (SLE), which is also characterized by an increased type I IFN production through pDCs, a Th2 immunological dominance and a substantial dysregulation of humoral immunity. Moreover, some human studies and murine experimental models suggested a pathogenic role for basophils in SLE, indeed[42].

However, in the landscape of all the innate immune cells implicated in the pathogenesis of type 1 AIP, the role of pDCs appears to be prominent. In addition to IFN-α, these cells resulted to be the main source of another important cytokine that has been implicated in type 1 AIP immunopathogenesis, namely IL-33. Importantly, IL-33 was also recognized to be an important inflammatory mediator in ordinary CP, whereby actually the main cell source is represented by the pancreatic acinar cells[9, 43]. IL-33 is able to activate Th2 cells and also group 2 innate lymphoid cells, which can further stimulate the production of IL-4, IL-5, and IL-13[44,45].

Finally, to complete the landscape of the innate immunity involvement in type 1 AIP, it is worth to mention M2-polarized macrophages, which were described in the pancreatic tissues of type 1 AIP patients affected with IgG4-RD[46]. Compared to classically activated or M1 macrophages (which are clearly pro-inflammatory, are polarized by lipopolysaccharide and/or Th1 cytokines, and mainly produce IL-1β, IL-6, IL-12, IL-23 and tumor necrosis factor-α), alternatively activated or M2 macrophages are polarized by Th2 cytokines and produce anti-inflammatory cytokines, such as IL-10 and TGF-β. Therefore, M2 macrophages have been implicated in angiogenesis and tissue repair, and are considered to exert an anti-inflammatory (and, for some aspects, tolerogenic effect) also in IgG4-RD and type 1 AIP[47,48].

Indeed, IL-10 and TGF-β are the main cytokines secreted by the antigen-induced or adaptive Tregs. In detail, IL-10 regulates the functions of many different immune cells, including macrophages themselves, dendritic cells, and both T and B lymphocytes. In detail, IL-10 is a potent inhibitor of antigen presentation by reducing the expression of major histocompatibility complex molecules and costimulatory molecules CD80 and CD86. Moreover, it inhibits the differentiation of dendritic cells (DCs) themselves from monocyte precursors and their further maturation. Finally, upon antigen presentation, IL-10 inhibits macrophage production of pro-inflammatory cytokines (IL-1, IL-6, IL-12, tumor necrosis factor-α), even though it does not seem to directly affect the Th1/Th2 balance[49,50].

These immune-pathogenic aspects of type 1 AIP and IgG4-RD may create a favorable immunological background in the affected pancreas for carcinogenesis promotion, as discussed in the next section.

PANCREATIC CANCER AND TYPE 1 AIP: IMMUNOLOGICAL CONSIDERATIONS

As already mentioned, the cumulative risk of pancreatic cancer in subjects with ordinary CP was reported to be 1.8% after 10 years and 4.0% after 20 years, and it is estimated to be more than 10 times higher in these patients than in healthy subjects[14,51].

Whereas carcinogenesis in the context of ordinary CP is then well established epidemiologically, this methodological approach has not clearly supported the association between AIP (in detail, type 1 AIP) and pancreatic cancer, but the numerous study limitations should prevent from ruling it out definitively, as it was previously discussed.

To start with, a few initial molecular findings should be considered. Some specific molecular alterations of pancreatic carcinogenesis may be functional to this discussion, such as K-ras mutations, which resulted to be present in > 90% of pancreatic cancers and, importantly, may occur at an early stage of this neoplastic process, namely at the PanIN stage[52]. For instance, Kamisawa *et al*[53] found that codon-12 mutation of K-ras was significantly more frequent in the pancreato-biliary regions of patients with AIP than in those affected with chronic alcoholic pancreatitis.

More recently, Kinugawa *et al*[54] also described some methylation abnormalities of tumor suppressor genes in AIP patients. Promoter region hyper-methylation with consequent gene silencing was reported for several genes in pancreatic cancer. In detail, among the 6 genes that these authors have investigated (because those were previously reported as methylated in pancreatic cancer), they found a statistically significant difference in terms of the TFPI2 (tissue factor pathway inhibitor 2) methylation ratio between specimens taken from AIP patients and those from pancreas resected for non-tumoral diseases. TFPI2 was recognized as a tumor suppressor gene, and a previous study described methylation abnormalities for this gene in 73% of pancreatic carcinomas, whereas those were completely absent in normal pancreas specimens[55].

However, further theoretical support and biological plausibility supporting a role for type 1 AIP as potential risk factors for pancreatic cancer, may derive from some immunological considerations.

Indeed, the role of the immune system and, in detail, the impairment of the immunological surveillance in the development and progression of cancer, is well supported. Both the innate and the adaptive immune system play an active role in this regard. Generally speaking, cytotoxic CD8+ T cells, Th1 cells, mature DCs, classically activated pro-inflammatory macrophages M1 and natural killer cells are variably described as the main effectors of this anti-cancer immunological surveillance[56,57]. Therefore, several aspects of the immunological environment created by and during

type 1 AIP (and, systemically, by IgG4-RD) may be favorable to cancer development in the pancreas.

First, the Th2 signature dominating these diseases, does not support cytotoxic CD8+ T cell responses[56]. Tassi *et al*[58] showed that patients affected with pancreatic cancer have a Th2 skewed immune response, which can impair the specific CD4+ T cells response against a tumor-associated antigen, namely CEA; moreover, this polarization was suggested to negatively affect the T CD8+ cell compartment as well.

Second, in type 1 AIP macrophages tend to switch to a M2 phenotype, characterized by the production of IL-10 and TGF-β, which can create a tolerogenic immunological environment in the pancreas. By the way, Th2 cells themselves produce IL-10, in addition to their hallmark cytokines, such as IL-4, IL-5 and IL-13[47]. As explained, these M2 macrophages are also characterized by poor antigen presenting ability rather than supporting lymphocyte activation, proliferation and cytotoxicity. Moreover, they are committed to promote tissue remodeling and, importantly, neo-angiogenesis. Finally, M2 macrophages also express inhibitory ligands, such as PD-L1, which is known to induce peripheral T cell tolerance. All these properties suggested an important role of M2 macrophage in reducing the anti-tumor immunity for several malignancies, including pancreatic cancer. Indeed, they represent the prevalent macrophage phenotype among so-called tumor-associated macrophages and have been thus implicated in the processes of tumoral growth and cell migration/metastasis [59-62]. Accordingly, the degree and type of M2 macrophages infiltration into the tumoral stroma resulted to be an independent prognostic factor in patients with pancreatic cancer[63].

Third, in addition to M2 macrophages, basophils were recently suggested to play a role in the pathophysiology of type 1 AIP, upon activation *via* TLR signaling[38,64]. As previously explained, these cells can strongly shift the immunological balance toward a more tumor-tolerogenic Th2-polarized tissue microenvironment. Therefore, in addition to some initial evidence that basophils may be directly implicated in type 1 AIP pathogenesis, this way basophils might indirectly promote the pancreatic tumorigenesis as well. Recently, De Monte *et al*[65] investigated the presence of basophils in in tumor-draining lymph nodes of patients affected with pancreatic cancer, which resulted to correlate with both Th2 inflammation and reduced patients' survival. In this study, the role of basophils in tumor develop-ment/progression was also supported through some observations in basophil-deficient murine experimental models. Therefore, basophils may favor the tumorigenesis and progression of pancreatic cancer by promoting both Th2 and M2 polariza-tion[66].

Interestingly, these novel findings might open to additional important associations between pancreatic cancer and autoimmune diseases other than type 1 AIP and IgG4-RD. As mentioned, Watanabe *et al*[9] provided an interesting immunopathological parallelism between IgG4-RD and SLE. Therefore, it is very interesting that a very recent meta-analysis by Seo *et al*[67] evidenced that SLE was associated with increased risk for pancreatic cancer. These authors speculated several mechanisms that may potentially explain this association (*e.g.*, chronic inflammation, excess autoantibody effect, metabolic alterations); however, based on the present discussion, basophils activation and, more in general, Th2-driven inflammation described in active SLE might be added to these speculations on the hypothetical pathophysiologic permissive mechanisms[68,69].

CONCLUSION

Currently, the association between (type 1) AIP and pancreatic cancer does not find any clear epidemiological support, even though it cannot be ruled out definitively due to the small number of participants in the available clinical studies and the numerous study limitations. Some immunological aspects characterizing type 1 AIP, such as Th2 dominance, M2 macrophage polarization and basophil implication, may lead to an immunological environment favorable to pancreatic carcinogenesis and/or tumor progression.

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MINIREVIEWS

Gut microbiota in obesity

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Abstract

Obesity is a major global health problem determined by heredity and environment, and its incidence is increasing yearly. In recent years, increasing evidence linking obesity to the gut microbiota has been reported. Gut microbiota management has become a new method of obesity treatment. However, the complex interactions among genetics, environment, the gut microbiota, and obesity remain poorly understood. In this review, we summarize the characteristics of the gut microbiota in obesity, the mechanism of obesity induced by the gut microbiota, and the influence of genetic and environmental factors on the gut microbiota and obesity to provide support for understanding the complex relationship between obesity and microbiota. At the same time, the prospect of obesity research related to the gut microbiota is proposed.

Key Words: Gut microbiota; Obesity; Dysbiosis; Genetics; Ecology

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Core Tip: Obesity is closely related to the gut microbiota. The study of the gut microbiome provides a basis for the reconstruction of the gut microbiota of obese patients. Here, we discuss the characteristics of the gut microbiota in obesity, the mechanism by which the gut microbiota induces obesity, and the relationships between genetic and environmental factors and the gut microbiota in obesity.

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INTRODUCTION

Obesity is a complex metabolic disorder caused by a variety of genetic and nongenetic factors (such as environmental factors). The World Health Organization defines obesity as having a body mass index (BMI) greater than 30, but the definition varies from country to country. In China, for example, a BMI of 28 or greater is considered obese. A comprehensive analysis shows that approximately one-third of the world's population are overweight, and approximately 10% are obese[1]. It is predicted that by 2030, the number of obese people worldwide will reach 1.12 billion[2]. The health risk of obesity has caused widespread concern and has become an important global health problem. Obesity not only manifests as changes in appearance but is also associated with lipid and glucose metabolism disorders, chronic inflammation, oxidative stress, and an increased risk of a variety of diseases, most notably cardiovascular disease, diabetes, and cancer[3,4]. In recent years, increasing evidence has shown that an imbalance in the gut microbiota may be a factor leading to obesity[5,6].

Up to 100 trillion symbiotic microbes live in the gut, called the gut microbiota, which comprises 10 times the number of cells in the body itself[7]. The gut microbiota relies on food residues that the human body does not digest, mucus secreted by the gut, and dead cells that are shed as nutrients to maintain its high population levels[8]. The active gut microbiota will produce a large number of physiologically active substances, including short-chain fatty acids, vitamins, and health-beneficial products such as anti-inflammatory, analgesic, and antioxidant products, along with potentially harmful products such as neurotoxins, carcinogens, and immunotoxins[9,10]. These products can enter the blood, directly regulate the expression of genes, and affect human immune and metabolic processes. Therefore, a healthy gut microbiota is essential for maintaining the body's metabolism and energy balance. An imbalance in the gut microbiota can cause metabolic disorders and increase central appetite, leading to obesity. This article reviews the research progress of the relationship between the gut microbiota and obesity.

LITERATURE SEARCH

PubMed (https://pubmed.ncbi.nlm.nih.gov/) was used for search with the following keywords: Obesity, gut microbiota, dysbiosis, energy absorption, appetite, fat storage, chronic inflammation, and circadian rhythm. More than 4000 published papers including 178 clinical trial related to gut microbiota in obesity from 2000 through 2021 have been searched.

RESULTS

After the search, we summarize some characteristics of the gut microbiota of obese patients. Many results suggest that an increased Firmicutes/Bacteroidetes ratio at the phylum level is an important feature of the gut microbiota in obesity. The family Christensenellaceae and the genera *Methanobacteriales*, *Lactobacillus*, *Bifidobacteria*, and *Akkermansia* are usually considered as probiotics, and their relative abundance is often inversely associated with obesity. Gut microbiota regulates obesity by regulating energy absorption, central appetite, fat storage, chronic inflammation, and circadian rhythms. Finally, the effects of genetic and environmental factors on gut microbiota in obesity are discussed.

THE HEALTHY GUT MICROBIOTA

The development of the Human Microbiome Project has promoted the in-depth study of the gut microbiota[11,12]. With the application of metagenomic and 16S ribosomal RNA gene sequencing, the diversity of the gut ecosystem has been widely studied, which enables us to accurately understand the composition and function of the gut microbiota[13]. So far, the gut microbes of 6457 taxa have been collected in the gutMEGA database[14]. The normal gut microbiota of the human body is mainly composed of *Firmicutes*, *Bacteroides*, *Proteus*, *Actinomycetes*, *Fusobacteria*, and *Verrucomicrobia*, among which *Firmicutes* and *Bacteroides* dominate[15]. The core functions of a healthy gut microbiota include the biodegradation of polysaccharides, the production

of short-chain fatty acids, the enrichment of specific lipopolysaccharides, and the production of vitamins and essential amino acids[16]. A healthy gut microbiota is generally highly diverse; conversely, the relative lack of diversity in the gut microbiota leads to diseases such as obesity[17]. Another sign of a healthy gut microbiota is dynamic equilibrium, which refers to its ability to resist perturbation and return to a healthy state, such as with antibiotic treatment[18]. Therefore, changes in the structure and metabolism of the gut microbiota will affect the body's physiological processes, such as nutrient absorption and energy metabolism.

ASSOCIATION OF THE GUT MICROBIOTA WITH OBESITY

The hypothesis that the gut microbiota may be a relevant environmental factor in obesity has led to the study of the gut microbiomes of obese individuals. The first evidence for a link between the gut microbiota and obesity was from studies of germ-free mice. Transplanting gut microbes from conventionally raised mice into germ-free mice increased the transplants' fat content and insulin resistance levels even with reduced food intake, which proved that gut microbes can increase the accumulation of adipose tissue in the host[19]. Furthermore, 16S rRNA gene sequencing showed that obesity may be associated with two dominant bacterial phyla: Firmicutes and Bacteroidetes. The gut microbiota of obese mice showed a 50% decrease in the abundance of Bacteroidetes and a proportional increase in Firmicutes[20]. Turnbaugh *et al*[21] further confirmed that the Firmicutes/Bacteroidetes ratio increased significantly in obese mice and proved that the capacity of the microbiota in obese mice to harvest energy from the diet was stronger. Similar phenomena occur in humans; for example, the proportion of Firmicutes in the gut of obese children was increased, and the proportion of Bacteroidetes was decreased[22]. A study of the Ukrainian population found that the Firmicutes/Bacteroidetes ratio increased with increasing BMI [23]. However, some other studies have found opposite results. Zhang *et al*[24] found that the difference in the abundance of *Bacteroidetes* was not significant between obese and normal people. A study comparing the gut microbiota of obese and healthy people based on the public database of the intestinal program in the United States analyzed the gut microbiota of 1655 healthy and 898 obese adults and found that the ratio of Firmicutes/Bacteroidetes in obese people was relatively low[25]. Therefore, more research is needed to explore the relationship between the gut microbiota and obesity.

Other works have associated obesity with specific bacteria, such as the family Christensenellaceae and the genera *Methanobacteriales*, *Lactobacillus*, *Bifidobacteria*, and *Akkermansia*. Recently, the family Christensenellaceae was found to be associated with weight loss, and its relative abundance was inversely related to host BMI[26]. *Akkermansia muciniphila* is a key bacterium for weight loss. Supplementation with *A. muciniphila* improves metabolic parameters in overweight and obese subjects[27]. *Lactobacillus* and *Bifidobacterium* are traditional probiotics that play an important role in the balance of the human intestinal microecology. Crovesy *et al*[28] summarized the effect of *Lactobacillus* on body weight in overweight subjects and found that the beneficial effects were species specific. The abundance of *Lactobacillus paracasei* (*L. paracasei*) was negatively correlated with obesity, while the abundances of *L. reuteri* and *L. gasseri* were significantly correlated with obesity. At present, evidence of *Bifidobacterium* resistance to obesity has been obtained from animal experiments. Following administration of *Bifidobacterium* in animal models of diet-induced obesity, it also showed a strain-dependent effect on obesity[29]. However, reduced *Bifidobacterium* abundance in the gut is associated with obesity[30]. Million *et al*[31] found that *M. smithii* and *B. animalis* were associated with normal weight, while *L. reuteri* was associated with obesity. These findings suggest that obesity-related microorganisms are species specific and that bacteria in the same genus may have opposite effects, which may be related to the complex metabolic mechanism of obesity.

The persons with obesity can be divided into two different types: Subcutaneous obesity and visceral obesity. As mentioned above, an elevated Firmicutes/Bacteroidetes ratio is a biomarker for obesity. However, the relative abundance of Firmutes in morbid obesity is positively correlated with brown adipocytes markers in subcutaneous adipose tissue, but not in visceral adipose tissue[32]. The browning of the white adipose tissue is conducive to maintaining a relatively healthy obesity phenotype, suggesting that the higher relative abundance of Firmutes may be beneficial for subcutaneous obesity. In an experiment of rapid weight loss through a very low calorie diet in obese postmenopausal women, a decrease abundance of *Roseburia* and increased *Christensenellaceae* (unknown genus) were found to be

positively correlated with several gene expression pathways, such as protein-amino acid N-glycation, in subcutaneous adipose tissue[33]. Probiotics play a positive role in visceral obesity. Increased *Akkermansia* population reduced body weight and visceral obesity in mice fed a high-fat/high-sugar diet[34]. The probiotic mixture including *L. acidophilus*, *L. rhamnosus*, *L. paracasei*, *Pediococcus pentosaceus*, *B. lactis*, and *B. breve* significantly reduced BMI and intrahepatic fat fraction in patients with nonalcoholic fatty liver disease after 12 wk of administration. Except *L. paracasei*, the abundance of the other five probiotics increased in the gut of the probiotic mixture group[35]. Tavella *et al*[36] proposed that the relevance of large numbers of *Christensenellaceae*, *Porphyromonadaceae* and *Rikenellaceae* in the elderly gut microbiota contributed to the reduction of visceral adipose tissue. Due to the lack of studies on gut microbiota specifically targeting subcutaneous obesity and visceral obesity, further studies are needed to determine whether there are significant differences in gut microbiota characteristics between patients with subcutaneous obesity and visceral obesity.

From the above analysis, the role of microorganisms in obesity is strain specific, as there are both beneficial and harmful bacteria within the same taxon. Therefore, it is difficult to classify obesity-related bacterial communities according to their taxonomic relationships. Recent studies have introduced the concept of a 'guild' into the study of the gut microbiota, defining a group of microorganisms that utilize similar resources or perform the same biological function as the same guild to identify potential clusters associated with specific disease phenotypes and identify candidate gut microbes that may contribute to human health and disease[37]. Using a clinical study of genetically obese children, the researchers found that the abundance of one strain of *E. coli* increased sharply after 30 d of eating a diet rich in nondigested carbohydrates, while the abundance levels of the other four strains decreased[37]. Therefore, guilds, as an important form of intermember organization in ecology, can better reflect the changes in specific responses of strains.

The diversity of the gut microbiota is another important factor related to obesity. Most studies have shown that the diversity and richness of the gut microbiome are reduced in obese subjects[38-40]. Wu *et al*[25] found that the α-diversity of obese people was significantly lower than that of healthy people, but there was no significant difference in the structure of the gut microbiota (β-diversity) between them. However, some studies believed that there was not necessarily a correlation between the diversity of the gut microbiota and diseases[41]. Strict statistical analysis showed that only in approximately 1/3 of the cases was there a significant relationship between bacterial diversity and "microbiota-related diseases". Since bacterial ecosystems may possess a considerable degree of stability (including resistance to disease), the impression seems to be that bacterial diversity is closely related to disease occurrence and progression[41]. Obesity, as a flora-related disease without specific pathogens, may be directly related to the dysbiosis of the bacterial ecosystem. Therefore, in the face of the dilemma of diversity research, ecological theory is needed for guidance. The association between gut microbiomes and obesity has been summarized in Table 1.

OBESITY MECHANISMS INDUCED BY THE GUT MICROBIOTA

Energy absorption

Studies have found that genetically obese mice consume more carbohydrates and protein through the gut microbiota to provide energy for the host[42,43]. In animal experiments, it was found that in the case of no differences in diet or weight of the mice, the total body fat of the germ-free mice colonized by the 'obese microbiota' increased significantly compared with those colonized by the 'lean microbiota'[21]. This finding indicates that the obese-type gut microbiota has an increased ability to absorb energy from the diet. Multiomics analysis showed increased lipid absorption in obese hosts. Colonization of *Clostridia* in germ-free mice downregulated the genes that control lipid absorption[44]. Therefore, the gut microbiota of obese patients can promote the absorption of energy, resulting in excessive accumulation of energy and increased weight gain.

The gut microbiota ferments hard-to-digest carbohydrates into short-chain fatty acids (SCFAs), which are either absorbed by the gut or excreted in feces. SCFAs are crucial for energy homeostasis regulation[45]. SCFAs are composed mainly of acetate, propionate, and butyrate. Acetate can produce beneficial effects on the energy metabolism of the host by secreting glucagon-like peptide-1, peptide YY, and other intestinal hormones, reduce systemic lipolysis and proinflammatory cytokine levels, and increase energy consumption and lipid oxidation[46]. Propionate promotes

Table 1 Association between gut microbiomes and obesity

Microbiota characteristics in obesity	Preclinical or clinical	Study subjects	Ref.
Firmicutes/Bacteroidetes ratio increased	Preclinical	Mice	Ley *et al*[20], Turnbaugh *et al*[21]
	Clinical	Childhood	Indiani *et al*[22]
	Clinical	Adult ukrainian population	Koliada *et al*[23]
The relative abundance of Christensenellaceae was inversely related to host BMI	Clinical	Human	Waters *et al*[26]
	Clinical	Postmenopausal women	Alemán *et al*[33]
	Clinical	Italian elderly	Tavella *et al*[36]
Increased *Akkermansia* population reduced body weight	Clinical	Human	Depommier *et al*[27]
	Preclinical	Mice	Anhê*et al*[34]
Lactobacillus paracasei decreased, while *Lactobacillus reuteri* and *Lactobacillus gasseri* increased	Clinical	Human	Crovesy *et al*[28], Million *et al*[31]
Bifidobacteria reduced	Preclinical	Rats	Waldram *et al*[30]
Methanobacteriales smithii and *Bifidobacterium* were associated with normal weight	Clinical	Human	Million *et al*[31]

BMI: Body mass index.

intestinal lipolysis and energy homeostasis in mice through the AMPK/LSD1 pathway [47]. Butyrate is the colon's main energy source, and intestinal epithelial cells derive most of their energy from the oxidation of butyrate. The increase in butyrate-producing bacteria in the gut microbiota increases the production of butyrate, thereby improving lipid metabolism through the butyrate-SESN2/CRTC2 pathway[48]. However, excessive butyrate may reduce the proportion of probiotics and reverse the metabolic effects[48]. In recent years, SCFAs have attracted special attention due to their beneficial effects on intestinal homeostasis and energy metabolism, but their role in obesity remains controversial. Higher concentrations of fecal SCFAs are associated with gut permeability, metabolic disorder markers, obesity, and hypertension[49]. Teixeira *et al*[50] found that fecal SCFAs in women were positively correlated with obesity, waist circumference, and other indicators of metabolic syndrome. Overall, SCFAs seem to be a double-edged sword. Although they can protect the host from diet-induced obesity, excessive SCFAs provide extra energy for the host, thus promoting obesity.

Central appetite

In recent years, the microbiota has emerged as one of the key regulators of gut-brain function. This gut-brain axis has received increasing attention in the study of the biological and physiological bases of obesity and its related diseases. The microbiota and the brain communicate with each other through a variety of pathways, including endocrine, immune, and neural pathways[51]. The gut microbiota affects the host's central nervous system through the gut-brain axis. The central nervous system can also affect the composition and structure of the gut microbiota. The gut microbiota influences food intake by regulating brain function in a number of ways, such as by contributing to the production of neuromodulators, such as serotonin, which plays an important role in regulating gastrointestinal function[52]. Lactate produced by *Lactobacillus* and *Bifidobacterium*, which acts as a substrate for neuron cells, can prolong satiety after a meal[53]. The gut microbiota also participates in the gut-brain axis by regulating gut hormones secreted by enteroendocrine cells. Peptide YY, pancreatic polypeptide, and glucagon-like peptide-1 (GLP-1) are gut-brain peptides that play important roles in information communication of the gut-brain axis. Peptide YY and pancreatic polypeptide are anorexia hormones secreted by the gut. GLP-1 lowers glucagon levels, slows gastric emptying, stimulates insulin synthesis, and reduces food intake [54,55]. Studies have found that the levels of peptide YY and GLP-1 in obese patients are significantly decreased. However, after fat reduction surgery, the levels of peptide YY and GLP-1 gradually increase[56]. Schéle *et al*[57] compared the gene expression

levels of food intake-regulating neuropeptides in germ-free and conventionally raised mice and found that the gut microbiota decreased the expression of two genes encoding the body fat-inhibiting neuropeptides Gcg and Bdnf, which may contribute to the induction of fat mass by the gut microbiota. The gut microbiota and its metabolites have been shown to stimulate gut satiety. This short-term regulation of gut satiety, which is associated with bacterial growth, can be combined with long-term appetite regulation controlled by neuropeptide energy circuits in the hypothalamus[58].

Fat storage

In 2004, researchers reported the ability of the gut microbiota to regulate fat storage [19]. The gut microbiota increases the absorption of glucose in the host's intestine and the content of glucose in the serum, thereby increasing the expression levels of two basic transcription factors, ChREBP (carbohydrate response element binding protein) and SREBP-1 (sterol regulatory element binding protein), which induce fat synthesis in the liver. Lipoprotein lipase (LPL) helps triglycerides enter the circulatory system from the liver, where they are absorbed by fat cells. Intestinal epithelial cells can produce Fiaf, an inhibitor of LPL. Fiaf is selectively inhibited in normal mouse intestinal epithelial cells, thereby increasing the host's energy storage. Aronsson *et al*[59] found that *L. paracasei* regulated ANGPTL4, a central player in fat storage regulation, using a mouse model with high-fat diet-induced obesity. *L. paracasei* could induce the expression of ANGPTL4, partly through the peroxisomal proliferator-activated receptors α and γ. ANGPTL4 inhibited LPL, leading to decreased fat storage. *L. paracasei*-colonized mice resisted high-fat diet-induced obesity[60]. *In vitro*, *L. paracasei* inhibits the Akt/mTOR pathway, indicating that several regulatory pathways are involved in different intracellular lipid accumulations mediated by *L. paracasei*.

Chronic inflammation

Chronic inflammation is one of the characteristics of metabolic disorders such as obesity[61]. Evidence shows that these disorders are characterized by the gut microbiota and its metabolites crossing the intestinal barrier, affecting various metabolic organs, such as the liver and adipose tissue, leading to chronic inflammation[62]. Through fecal microbiota transplantation, the microbiota of normal mice was transplanted to chronic colitis mice. Fecal microbiota transplantation could reduce colitis in mice with chronic intestinal inflammation by regulating the expression of proinflammatory genes, antimicrobial peptides and mucin[63]. Lipopolysaccharide (LPS), an endotoxin, has been shown to be expressed at elevated levels in obesity and adipose tissue inflammation[64]. LPS binds to Toll-like receptor 4 on immune cells, thereby activating a proinflammatory cascade in the gut[65]. Studies have shown that increased levels of butyrate-producing Ruminococcaceae and Lachnospiraceae lead to reduced levels of members of the LPS family S247, thereby reducing chronic low-grade inflammation[66]. The gut microbiota can also help prevent high-fat diet-induced intestinal barrier dysfunction by inhibiting cannabinoid receptor type 1[66]. The endocannabinoid system is a major regulator of fat synthesis in the gut and fat cells, and its activation can increase appetite and food intake. In addition, the effects of the consumption of antibiotics on the microbiota are sufficient to block the obesity protection phenotype, further indicating the roles of the microbiota in chronic inflammation and obesity.

SCFAs play a key role in the interaction between the gut microbiota and the activation or inhibition of the inflammatory cascade. Butyrate is an anti-inflammatory metabolite known to inhibit pathways leading to the production of pro-inflammatory cytokines[67]. Through epigenetic interactions, butyrate stimulates adipoliolysis and mitochondrial oxidative phosphorylation, thereby achieving greater energy consumption and preventing obesity[68]. As stated above, butyrate has also been shown to reduce LPS in the gut, thereby reducing LPS-related effects. Another SCFA, acetate, has a more controversial role in chronic inflammation and obesity. On the one hand, acetate can be used as a substrate for cholesterol synthesis, thus helping to raise serum cholesterol levels, which can increase the risk of obesity[69]. On the other hand, acetate has been reported to suppress appetite and reduce the risk of obesity[70]. Acetate is an important metabolite, and its role in the regulation of the gut microbiota and obesity is quite complicated. Therefore, further research is needed to clarify the exact mechanisms of the interactions between SCFAs and chronic inflammation, microbiota composition, and obesity.

Circadian rhythm

The gut microbiota has been shown to influence host circadian rhythms in a diet-

dependent manner[71]. The interruption of the circadian rhythm may lead to an increase in the incidence of obesity[72]. Microorganisms regulate lipid uptake and storage by regulating the circadian transcription factor NFIL3. The study also found that the ILC3-STAT3 signaling pathway is a key molecular pathway for the interaction between the microbiota and the circadian clock. A recent study has shown that the gut microbiota programs rhythmic histone acetylation through HDAC3 (histone deacetylase 3) expression in intestinal epithelial cells so that *Cd36* (lipid transporter gene) transcription becomes rhythmic, which promotes lipid absorption and obesity[73]. The recruitment of histone modifiers to chromatin is a key mechanism for generating rhythm. Therefore, the gut microbiota regulates the circadian rhythm through HDCA3. Feeding rhythms are considered to be a potential regulator of circadian rhythms and the gut microbiota. Studies have shown that time-restricted feeding reduces the harmful effects of a high-fat diet by regulating the circadian rhythm of the gut microbiota[74,75]. The feces of individuals experiencing jet lag and individuals with regular schedules were transplanted into germ-free mice. As a result, the transplanted mice that experienced jet lag developed obesity and insulin resistance. Due to impaired feeding rhythms, jet lag induces microbiota dysbiosis, promoting glucose intolerance and obesity[76].

Metabolites of the gut microbiota can also affect the host rhythm system. Bile metabolism is an example of the rhythmic interaction between host and gut microbes. Microbial bile salt hydrolases are related to the regulation of the circadian clock and lipid metabolism-related genes[77]. Gut bacteria such as Lachnospiraceae, Clostridiaceae, Ruminococcaceae, *Lactobacillus*, *Bacteroides*, and *Bifidobacterium* play a role in the biotransformation of bile salts[78]. SCFAs directly regulate the expression of clock genes in liver cells. Treatment with acetate or butyrate regulated the expression of the clock genes *Per2* and *Bmal1* in hepatic cells synchronized with serum shock[71]. Another study suggested that SCFAs induce indirect regulation of the internal circadian clock because no phase change in the peripheral circadian clock was detected in cultured fibroblasts or cultured liver slices[79].

The mechanism of obesity induced by the gut microbiota is summarized in Table 2. Dysbiosis of the microbiota causes metabolic disorders and promotes the occurrence and development of obesity through the direct interaction between the microbiota and local tissues or the indirect interaction between metabolites and remote organs (Figure 1). Although we can often detect specific different microorganisms between obese and normal individuals and verify the role of the bacteria in obesity through germ-free mouse experiments, more attention should be paid to ecological theory applications in relation to the gut microbiota as a group. The gut microbiota affects appetite, energy absorption, fat storage, circadian rhythm, and chronic inflammation, leading to obesity. Therefore, targeted reconstruction of the gut microbiota structure, such as through fecal bacterial transplantation, is one of the means of treating obesity.

MICROBIOTA IN OBESITY: LINKS WITH GENETICS AND TRANSMISSION

Obesity is the result of interactions between genetic and environmental factors. It is not surprising that host genetics shapes the gut microbiota. In fact, several genetic variations explain differences in the composition and diversity of the gut microbiota in obese people. Whole-genome association was used to determine the relationship between the genetic variation of twins and different species of bacteria. More than 12 health-related gut microorganisms were identified[80]. These microorganisms are environment-acquired, but genes also have a certain impact on them, such as the associations between *Bifidobacterium* and the lactase gene locus[80] and AMY1-CN (CN variation of the AMY1 Locus, which encodes salivary amylase) as a genetic factor related to the composition and function of the microbiome[81]. The abundance of resistant starch-degrading microbes in the gut microbiota of high-AMY1-CN subjects increased, which resulted in a higher incidence of obesity after the microbes were transferred to germ-free mice[81]. In another study, host kinship indicated the structural similarity of the gut microbiota in wild house mice. The exon sequencing results of host genes were compared with the microbiota structure, and 20 host genes were found to be related to the diversity or abundance of the gut microbiota, including a cytokine IL12A gene with three nonconsensus mutations. Among the 20 related genes, there are a large number of homologous genes of human-microbiota interactions, including genes related to immune regulation and obesity[82]. Furthermore, a human genome-wide association analysis revealed that there was an association between an obesity-related genus (*Akkermansia*) and a variant near the phospholipase

Table 2 Mechanism of obesity induced by gut microbiota

Effect	Microbiota characteristics	Mechanism	Ref.
Increased energy absorption	Expansion of *Desulfovibrio* and loss of Clostridia	Elevated the expression of genes that control lipid absorption such as CD36	Petersen *et al* [44]
Extra energy for the host	The inverse association between fecal SCFAs and gut microbiota diversity; Faecalibacterium prausnitzii, Roseburia faecis, and other Clostridiales increased; Akkermansia muciniphila, Alistipes finegoldii, Bacteroides, Christensenellaceae, Methanobrevibacter, and Oscillospira decreased	Excessive SCFAs	de la Cuesta-Zuluaga *et al* [49]
Increased appetite	A community dominated by members of the Clostridial clusters XIVa and IV	The levels of peptide YY and GLP-1 in obese patients decrease significantly	Wu *et al*[54], Salehi *et al* [55], Federico *et al*[56]
Decreased Fat storage	Germ free mice colonized with *Lactobacillus paracasei*	Increase the expression of ANGPTL4, and inhibit LPL, leading to decreased fat storage	Aronsson *et al* [59], Tazi *et al* [60]
Increased fat storage	Transplanting gut microbes from conventionally raised mice into germ-free mice	Increasing the expression of ChREBP and SREBP-1, Fiaf is inhibited, activate LPL, help triglycerides enter the circulatory system from the liver	Bäckhed *et al* [19]
Decreased chronic inflammation	Increase levels in the butyrate-producing bacteria such as Ruminococcaceae and Lachnospiraceae	Inhibit pathways leading to the production of pro-inflammatory cytokines; Stimulate adipoliolysis and mitochondrial oxidative phosphorylation, thereby achieving greater energy consumption; Reduce LPS, thereby reducing chronic low-grade inflammation	Kang *et al*[66], Lührs *et al*[67], Jia *et al*[68]
Interruption of circadian rhythm	Bile salts biotransformation bacteria such as Lachnospiraceae, Clostridiaceae, Ruminococcaceae, Lactobacillus, Bacteroides, and Bifidobacterium	Regulate transcription of key genes involved in circadian rhythm (*Dbp, Per1/2*) and lipid metabolism (*Pparγ, Angptl4*)	Joyce *et al*[77], Parkar *et al*[78]

SCFAs: Short-chain fatty acids; LPL: Lipoprotein lipase; LPS: Lipopolysaccharide.

D1 gene, which is related to BMI[83].

In addition, the gut microbiota can be transmitted from mother to child. In a passage experiment with germ-free mice, it was found that the gut microbiota of these mice was very stable. These microbiota types accounted for the majority of the gut microbiota of the mice in the normal environment, which proved that most of the gut microbiota of mice came from vertical transmission from the mother[84]. Studies have shown that the microbiota exists in the placenta, amniotic fluid, umbilical cord blood, and meconium, and maternal microorganisms may play an important role in the establishment of the child's microbiota[85]. Therefore, maternal obesity during pregnancy is accompanied by dysbiosis of the gut microbiota and metabolic disorders, and the maternal microbiota is passed on to the child, which can lead to metabolic disorders in the child. Obesity-related bacteria are present in the placenta and amniotic fluid in obese mothers. The fetus swallows these bacteria, resulting in gut microbiota colonization in the uterus. Studies have confirmed that maternal obesity is related to dysbiosis of the gut microbiota in offspring[86]. Therefore, the increased risk of obesity in children with obese mothers can be partly explained by the spread of gut microbes from obese mothers to their offspring.

MICROBIOTA IN OBESITY: LINKS WITH ENVIRONMENTAL FACTORS

Although genes play important roles in the gut microbiota, environmental factors have a more significant impact. A study of 1000 Israelis from all over the world who have very similar eating habits and lifestyles found that their ancestry was not related to the microbiome. The overall heritability of the microbiome may be less than 2%, and more than 20% of the variation in the microbiome is related to diet, drugs, and anthropometric measurements[87].

Diet is one of the most important factors inducing obesity. The eating habits of developed countries and regions have gradually become characterized by high fat and high sugar consumption, which has contributed to the gradual increase in obesity. Gut microbes depend on the host diet to survive and harvest energy, and dietary changes

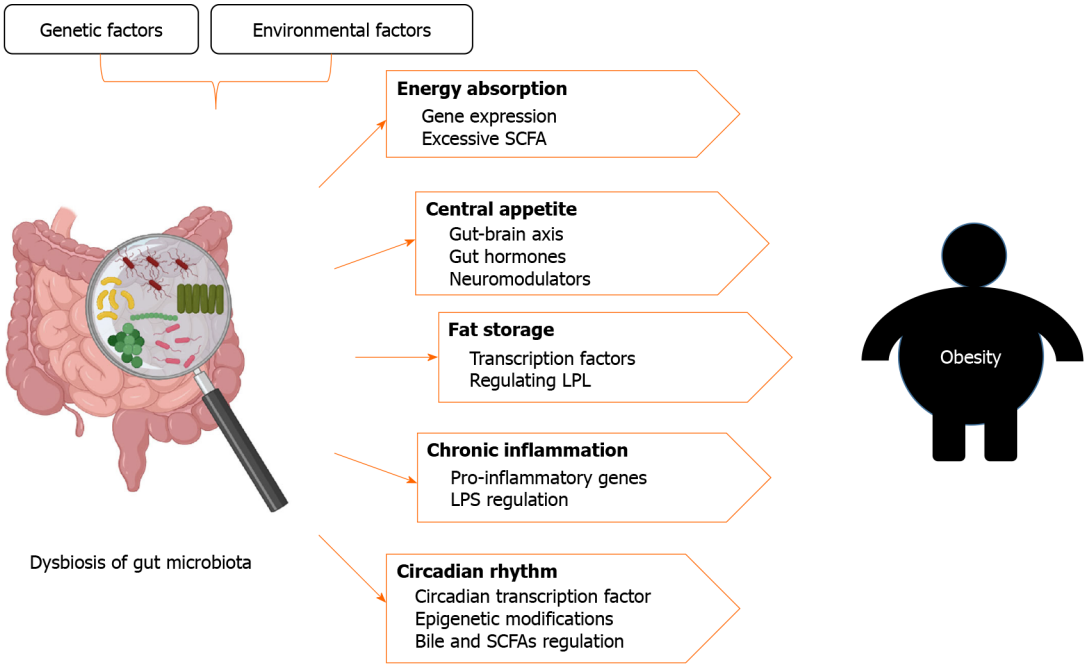


Figure 1 Gut microbiota and obesity. Both genetic and environmental factors can cause dysbiosis of the gut microbiota. Dysbiosis can increase energy absorption through changes in gene expression and excessive accumulation of short-chain fatty acids (SCFAs); improve central appetite through gut-brain axis, gut hormones, and neuromodulators; regulate fat storage through transcription factors and lipoprotein lipase; cause chronic inflammation through regulation of inflammatory gene expression and lipopolysaccharide; and disrupt the circadian rhythum by affecting the circadian transcription factors, epigenetic modifications, and the synthesis of bile and SCFAs. These factors appear to increase susceptibility to obesity. The figure was created with BioRender.com. SCFA: Short-chain fatty acids; LPL: Lipoprotein lipase; LPS: Lipopolysaccharide.

have a great impact on the gut microbiota. For example, the abundance of *Bacteroidetes* decreased while that of *Firmicutes* and *Proteobacteria* increased in mice fed a high-fat diet. These changes were observed in mice resistant to weight gain, suggesting that dietary fat has a direct effect on the microbiome[88,89]. Sleep disturbance is another cause of obesity. Lack of sleep leads to disrupted circadian rhythms, which can affect the gut microbiome and contribute to obesity. Chronic sleep fragmentation resulted in increased food intake and reversible changes in the gut microbiota, with increases in the abundance levels of Lactobacillaceae and *Ruminococcus* and a decrease in the abundance of Lactobacillaceae. These factors lead to systemic and visceral white adipose tissue inflammation and changes in insulin sensitivity[90]. Stress activates genes that affect metabolism and promotes the consumption of sweet and fatty foods, thus increasing appetite and contributing to obesity[91]. Stress significantly affects the microbiota-gut-brain axis at all stages of life[51]. During stress, independent of diet, the α-diversity of the gut microbiota increased, 50% of identified genera changed, and the abundance of Bacteroides decreased, while the abundance levels of less dominant taxa increased[92]. In addition, unhealthy lifestyle choices (sedentary, lack of exercise), emotional disorders, and drugs can also contribute to obesity. In short, as a "migrant", the gut microbiota itself is obtained from the environment; thus, environmental factors have a greater impact on it and can affect the occurrence and development of obesity.

STUDY LIMITATIONS

Although there are many factors contributing to obesity, the link between gut microbiota and obesity is widely accepted. In this review, the relationship between gut microbiota and obesity and the mechanism of gut microbiota inducing obesity are provided. However, the researches on gut microbiota in obesity are limited by the underrepresentation of individuals. This drawback limits the generality of these findings. Because of the specificity of the strains, microbes from the same genus may have opposite effects on obesity, which partly explains some of the contradictory results. In addition, this review only discusses the occurrence and development of obesity from the perspective of gut microbiota, and does not make further analysis of the relevant treatment plan.

CONCLUSION

Dysbiosis of the gut microbiota has been shown to be closely linked to obesity. Many gut microorganisms have been identified to be related to obesity. They induce the occurrence and development of obesity by increasing host energy absorption, increasing central appetite, enhancing fat storage, contributing to chronic inflammation, and regulating circadian rhythms. Due to the complexity and diversity of the gut microbiota, the mechanism by which the gut microbiota induces obesity still needs to be further studied. Obesity is the result of a combination of genetic and environmental factors. Data analysis based on larger samples to clarify the mechanism of the association between the gut microbiota and obesity, functional group studies of ecological significance to identify potential pathogenic members of the gut microbiota associated with obesity, and specific microbiota management for obese individuals will be the focus of future research.

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ORIGINAL ARTICLE

Basic Study

Zinc oxide nanoparticles reduce the chemoresistance of gastric cancer by inhibiting autophagy

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Abstract

BACKGROUND

Gastric cancer (GC) is a common malignancy that results in a high rate of cancer-related mortality. Cisplatin (DDP)-based chemotherapy is the first-line clinical treatment for GC therapy, but chemotherapy resistance remains a severe clinical challenge. Zinc oxide nanoparticle (ZnO-NP) has been identified as a promising anti-cancer agent, but the function of ZnO-NP in GC development is still unclear.

AIM

To explore the effect of ZnO-NP on chemotherapy resistance during GC progression.

METHODS

ZnO-NP was synthesized, and the effect and underlying mechanisms of ZnO-NP on the malignant progression and chemotherapy resistance of GC cells were analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, colony formation assays, transwell assays, wound healing assays, flow cytometry, and Western blot analysis in GC cells and DDP-resistant GC cells, and by tumorigenicity analyses in nude mice.

RESULTS

Our data revealed that ZnO-NP was able to inhibit proliferation, migration, and invasion and induce apoptosis of GC cells. Meanwhile, ZnO-NP significantly reduced the half maximal inhibitory concentration (IC_{50}) of DDP for the inhibition of cell proliferation of DDP-resistant SGC7901/DDP cell lines. Autophagy was increased in DDP-resistant GC cells, as demonstrated by elevated light chain 3-like protein 2 (LC3II)/LC3I and Beclin-1 expression and repressed p62 expression in SGC7901/DDP cells compared to SGC7901 cells. Mechanically, ZnO-NP inhibited autophagy in GC cells and treatment with DDP induced autophagy, which was reversed by ZnO-NP. Functionally, ZnO-NP attenuated the tumor

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growth of DDP-resistant GC cells *in vivo*.

CONCLUSION

We conclude that ZnO-NP alleviates the chemoresistance of GC cells by inhibiting autophagy. Our findings present novel insights into the mechanism by which ZnO-NP regulates the chemotherapy resistance of GC. ZnO-NP may serve as a potential therapeutic candidate for GC treatment. The potential role of ZnO-NP in the clinical treatment of GC needs clarification in future investigations.

Key Words: Gastric cancer; Progression; Chemoresistance; Zinc oxide nanoparticle; Autophagy; MTT assays

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Core Tip: We show that zinc oxide nanoparticle (ZnO-NP) reduces the chemoresistance of gastric cancer (GC) cells by inhibiting autophagy. Our findings provide innovative insights into the scenario in which ZnO-NP mediates chemotherapy resistance in GC. ZnO-NP may serve as a potential therapeutic candidate for GC treatment.

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INTRODUCTION

Gastric cancer (GC) is the second most common cause of cancer-related mortality globally[1]. Chemotherapy is the preferred treatment for advanced-stage GC patients, in which oxaliplatin, 5-fluorouracil (5-FU), cisplatin (DDP) are first-line therapies[2-4]. Although advancements have been made in chemotherapy effectiveness, the survival rate remains unsatisfactory due to chemotherapy resistance[5,6], which significantly limits the efficiency of GC treatments[7]. The molecular mechanisms underlying the regulation of GC chemotherapy resistance are complicated and remain poorly understood[8]. Accordingly, therapeutic strategies for the attenuation of chemotherapy resistance are urgently needed.

Autophagy is a process in which cellular contents, such as dysfunctional organelles and large protein groups, are transported to lysosomes for degradation and reuse[9]. Autophagy sustains cellular homeostasis and limits cellular damage under multiple stresses[10]. Autophagy has dual roles in cancer progression[11]. In some cases, autophagy induces cancer cell survival by recovering intracellular contents and increasing energy generation to reach the high metabolic requirements of cancer cells. In other contexts, autophagy inhibits cell imbalance and damage to attenuate tumorigenesis[12]. Autophagy is closely associated with the chemotherapy resistance of GC cells, and inhibition of autophagy relieves chemoresistance[13,14]. Autophagy is correlated with cell differentiation and tumor development in GC[15]. Thus, autophagy-related factors may be promising prognostic indicators of advanced GC[16].

Nanoparticles (NPs) are currently applied in multiple biomedical fields including bone regeneration, wound healing, bio-imaging, and targeted-drug transmission systems[17-21]. The conversion of material to nanoscale regularly leads to changes in chemical, physical (electric and magnetic), morphological, and structural properties [19,21]. These modifications permit NPs to cooperate with different biomolecules to affect certain responses[19,21]. Due to the unique surface and size properties, NPs have numerous benefits that enable them to serve as potential anti-tumor therapeutics [22]. Among them, metal zinc oxide NPs (ZnO-NP) have various properties and are widely used as critical components of many biomedical and cosmetic applications including sunscreen, foot care, and ointments[23]. ZnO-NPs exhibit antibacterial activities[24,25], and are also extensively used in drug targeting due to their biocompatibility[26]. However, the effect of ZnO-NP on the chemotherapy resistance of GC

cells remains unknown.

In this study, we focused on the impact of ZnO-NP on chemotherapy resistance of GC cells. We revealed the innovative role of ZnO-NP in repressing chemoresistance and reducing GC progression *via* inhibition of autophagy.

MATERIALS AND METHODS

Cell culture and treatment

The SGC7901, BGC823, and SGC7901/DDP cell lines were maintained in the lab. The cells were incubated in an incubator of 5% CO_2 and 37 °C in RPMI 1640 medium (Hyclone, Logan, UT, United States) with fetal bovine serum (10%; Hyclone), streptomycin (0.1 mg/mL; Hyclone) and penicillin (100 units/mL; Hyclone). DDP was obtained (Sigma, St. Louis, MO, United States) and used at the indicated doses.

ZnO-NP synthesis

A total of 0.5 mol/L zinc nitrate was plated to 1 mol/L sodium hydroxide solution, followed by continuous stirring (15 min). The white precipitate formed was washed and centrifuged, followed by repeated distilling of H_2O. The collected white powder [Zn(OH)2] was dried in a hot air oven at 60 °C. During drying, Zn2[24] was entirely converted to ZnO. Then, the dried ZnO powder was annealed at 500 °C for 3 h to convert to ZnO-NP. To analyze the effect of ZnO-NP on the malignant progression and chemotherapy resistance, the GC cells were treated with ZnO-NP at a dose of 5 μg/mL.

MTT assay

Cell viability was assessed by MTT assays at the indicated times in 6-well dishes. Briefly, the MTT solution (Solarbio, Beijing, China) was added to the cells and cultured at 5% CO_2 and 37 °C for 4 h. Next, dimethyl sulfoxide (100 μL, 10 min; Sigma) was used to terminate the reaction. Cell viability was analyzed at an absorbance of 490 nm with a microplate reader (Thermo Fisher Scientific, Waltham, MA, United States).

Colony formation assays

About 1×10^3 cells were plated in 6-well dishes and cultured in RPMI 1640 medium at 5% CO_2 and 37 °C. After 2 wk, cells were washed with phosphate-buffered saline (PBS) for about 30 min and dyed with 1% crystal violet dye, after which the number of colonies was calculated.

Transwell assays

Transwell assays were conducted to analyze the invasion and migration of melanoma cells by using a Transwell plate (Corning, New York, NY, United States) according to the manufacturer's instructions. Briefly, the upper chambers were plated with about 1×10^5 cells. Then cells were fixed in 4% paraformaldehyde and dyed with crystal violet. The invaded and migrated cells were recorded and calculated.

Wound healing assay

Cells were plated in a 24-well plate at a density of 3×10^5 cells/well and cultured overnight to reach full confluence as a monolayer. A 20 μL pipette tip was applied to slowly cut a straight line across the well. Then the well was washed three times with PBS, and changed to serum-free medium, followed by continued culture. The wound healing percentage was calculated.

Analysis of cell apoptosis

Cell apoptosis was measured using the Annexin-V-Fluorescein Isothiocyanate Apoptosis kit (BD Biosciences, San Jose, CA, United States) based on flow cytometry analysis using the FACSCalibur flow cytometer, followed by quantification with FlowJo software.

Western blot analysis

Total proteins were extracted from the cells using radioimmunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA, United States) and quantified using the BCA Protein Quantification Kit (Abbkine Scientific Co., Ltd., Palo Alto, CA, United States). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene fluoride membranes (Millipore,

Burlington, MA, United States), followed by incubation with 5% milk and with primary antibodies at 4 °C overnight. The membranes were incubated with the corresponding secondary antibodies (Boster Biotechnology, Wuhan, China) for 1 h at room temperature, followed by protein detection by chemiluminescence (Beyotime Biotechnology, Shanghai, China). The primary antibodies used in this study were against light chain 3B (LC3B), p62, Beclin-1, and β-actin (all from Abcam, Cambridge, MA, United States).

Analysis of tumorigenicity in nude mice

The tumor growth of GC cells *in vivo* was evaluated in Balb/c nude mice (4-week-old, male, $n = 5$). About 1×10^7 SGC7901/DDP cells were subcutaneously injected in the mice. After 5 d, we measured tumor growth every 5 d. We sacrificed the mice after 30 d, and tumors were scaled. Tumor volume (V) was determined by estimating the length (L) and width (W) with calipers and measured with the formula $(L \times W^2) \times 0.5$. Animal care and methods were authorized by the Animal Ethics Committee of Nantong Third People's Hospital (Jiangsu, China).

Statistical analyses

Data are presented as mean ± SD, and statistical analyses were performed with GraphPad Prism 7. The unpaired Student's *t*-test was applied for comparing two groups, and one-way analysis of variance was applied for comparing among multiple groups. $^aP < 0.05$ was considered statistically significant.

RESULTS

ZnO-NP inhibits proliferation and induces the apoptosis of GC cells

To investigate the effect of ZnO-NP on GC cells, SGC7901 and BGC823 cells were treated with ZnO-NP or an equal volume of saline. The cell viability was significantly inhibited by ZnO-NP treatment of the cells (Figure 1A and B). Consistently, ZnO-NP markedly reduced the colony numbers of SGC7901 and BGC823 cells (Figure 1C and D). Moreover, the apoptosis of SGC7901 and BGC823 cells was enhanced by treatment with ZnO-NP (Figure 1E and F), suggesting that ZnO-NP is able to inhibit proliferation and induce apoptosis of GC cells.

ZnO-NP reduces the invasion and migration of GC cells

Next, the role of ZnO-NP in regulating the invasion and migration was evaluated. Transwell assays revealed that the invasion and migration of BGC823 and SGC7901 cells were significantly attenuated upon treatment with ZnO-NP (Figure 2A and B). In addition, wound healing assays demonstrated that ZnO-NP markedly enhanced the wound proportion in SGC7901 and BGC823 cells (Figure 2C and D), indicating that ZnO-NP alleviates the migration and invasion of GC cells.

ZnO-NP attenuates the chemotherapy drug resistance of GC cells

We further explored the impact of ZnO-NP on the DDP of GC cells. Significantly, treatment with ZnO-NP notably reduced the IC_{50} value of DDP for inhibition of cell proliferation in DDP-resistant SGC7901/DDP cell lines (Figure 3A). Furthermore, DDP enhanced the apoptosis of SGC7901/DDP cells, which was markedly reinforced by ZnO-NP treatment (Figure 3B and C), suggesting that ZnO-NP attenuates the DDP resistance of GC cells.

Autophagy is increased in chemotherapy-resistant GC cells

Next, we were interested in the correlation of autophagy with the DDP resistance of GC cells. For this purpose, we analyzed the expression of autophagy markers including LC3B-II, LC3B-I, Beclin-1, and p62 in SGC7901 and SGC7901/DDP cell lines. Our data showed that the expression ratio of LC3II/LC3I and levels of Beclin-1 were elevated while p62 expression was inhibited in SGC7901/DDP cells compared with those in SGC7901 cells (Figure 4), indicating that autophagy is increased in DDP-resistant GC cells.

ZnO-NP inhibits autophagy in GC cells

We investigated the effect of ZnO-NP on autophagy in GC cells. We found that the treatment of ZnO-NP inhibited LC3II/LC3I and Beclin-1 levels but promoted p62 expression in SGC7901 and BGC823 cells (Figure 5A-H). Moreover, our data revealed

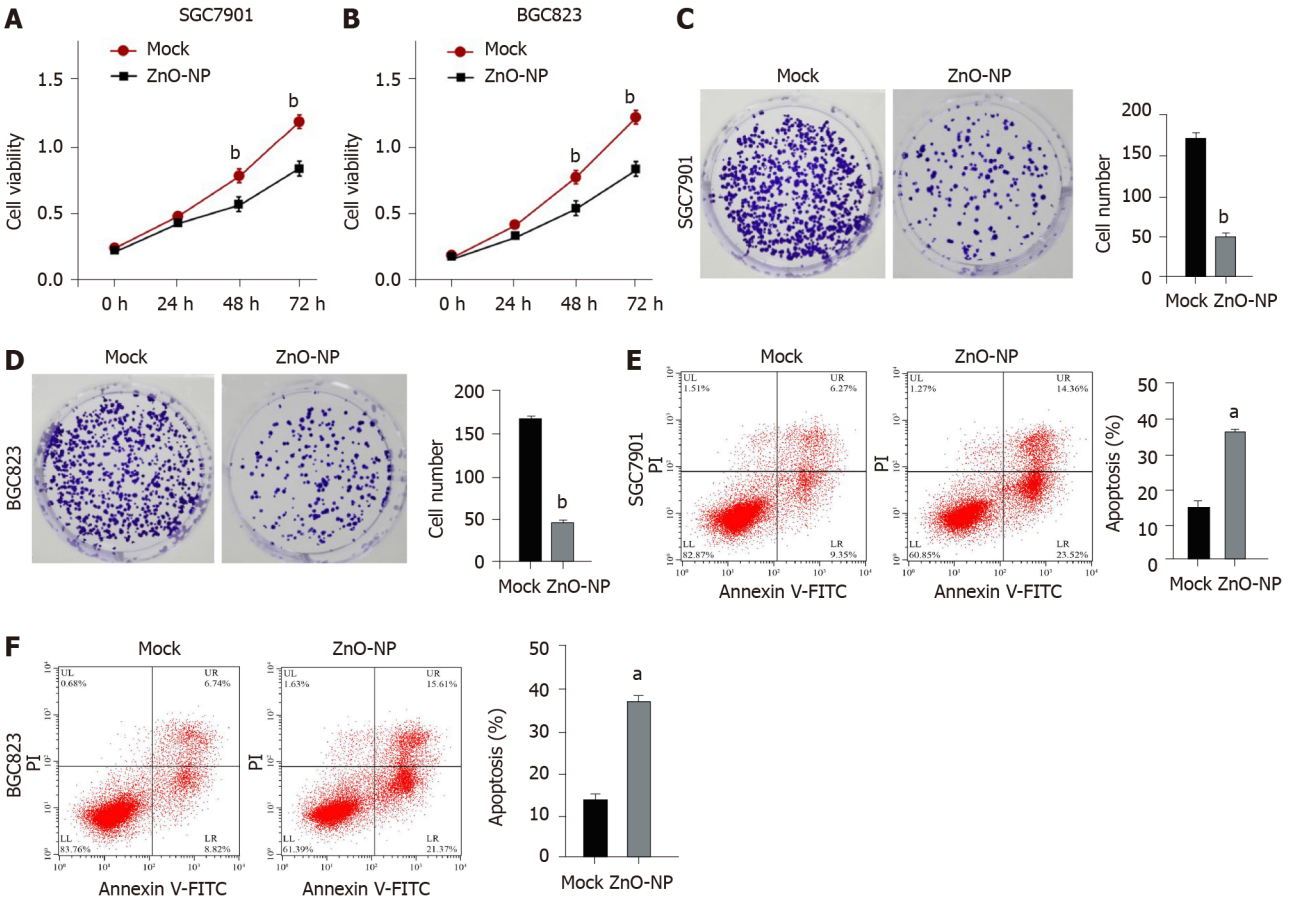


Figure 1 Zinc oxide nanoparticle inhibits proliferation and induces apoptosis of gastric cancer cells. SGC7901 and BGC823 cells were treated with the zinc oxide nanoparticle (ZnO-NP, 5 μg/mL) or an equal volume of saline. A and B: Cell viability was analyzed by the MTT assay; C and D: Cell proliferation was assessed by the colony formation assay; E and F: Cell apoptosis was measured by flow cytometry. Data are presented as the mean ± SD. Statistically significant differences are indicated: $^{a}P < 0.05$; $^{b}P < 0.01$. FITC: Fluorescein isothiocyanate.

that treatment with DDP induced the expression ratio of LC3II/LC3I and the levels of Beclin-1 and decreased the p62 expression in the cells; treatment with ZnO-NP reversed this effect (Figure 5I and J), indicating that ZnO-NP can inhibit autophagy in GC cells.

ZnO-NP attenuates chemotherapy drug resistance by inhibiting the autophagy of GC cells

We explored whether ZnO-NP modulated the DDP resistance of GC cells by regulating autophagy. Treatment with DDP reduced the viability of SGC7901 and BGC823 cells, while ZnO-NP or the autophagy inhibitor 3-methyladenine (3-MA) was able to further inhibit the phenotype (Figure 6A and B). Moreover, the cell apoptosis of SGC7901 and BGC823 cell lines was induced by DDP treatment, in which the treatment of ZnO-NP or 3-MA could reverse this effect in the cells (Figure 6C and D), suggesting that ZnO-NP attenuates chemotherapy drug resistance by inhibiting autophagy of GC cells.

ZnO-NP reduces the tumor growth of chemoresistant GC cells in vivo

Next, the effect of ZnO-NP on DDP-resistant GC cell growth *in vivo* was assessed by tumorigenicity analysis. The tumor growth of SGC7901/DDP cells was attenuated by ZnO-NP treatment of nude mice (Figure 7), indicating that ZnO-NP is able to reduce the tumor growth of chemoresistant GC cells *in vivo*.

DISCUSSION

The chemotherapy resistance of GC patients serves as a severe clinical challenge[1]. ZnO-NP has potential anti-tumor activities, but its role in modulating the chemo-

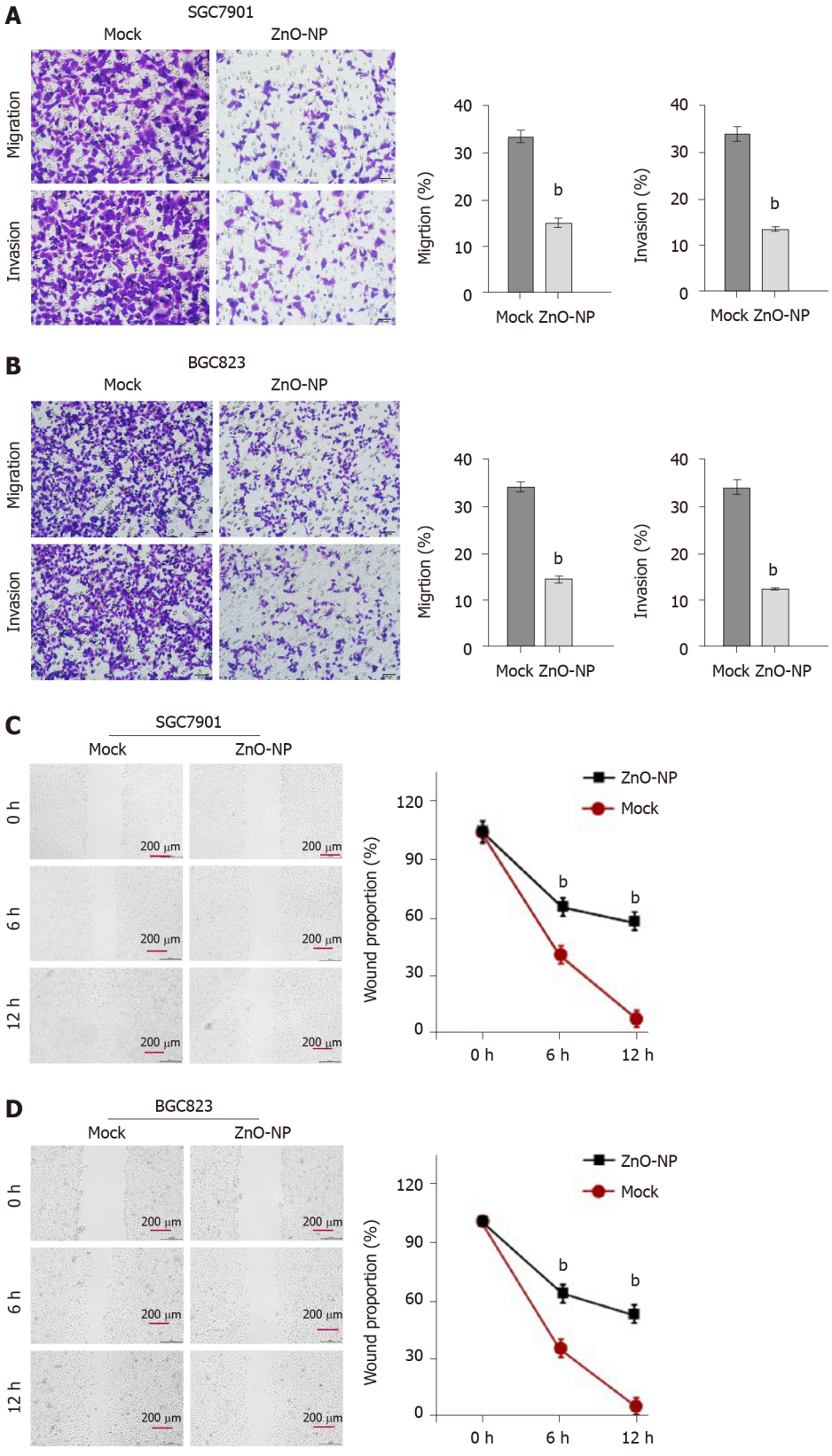
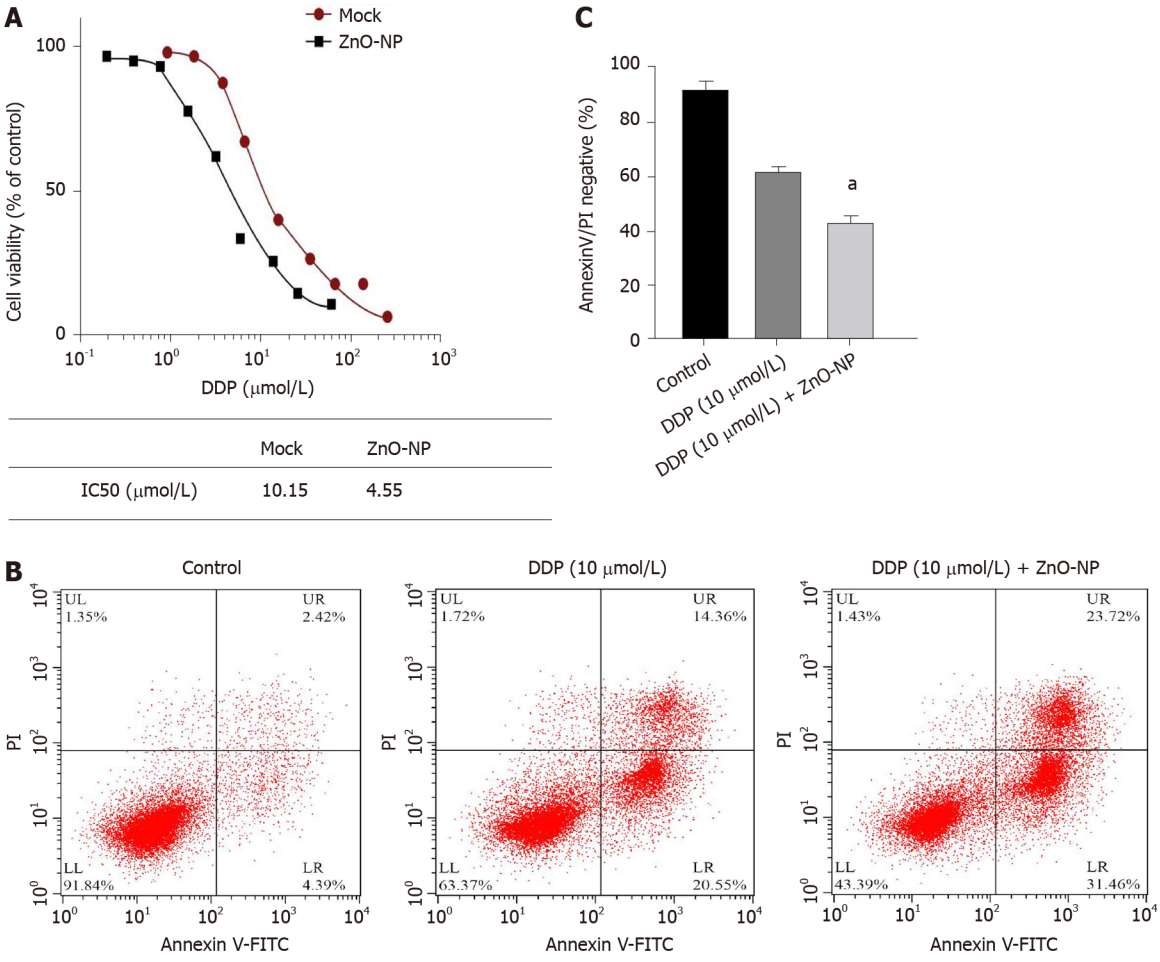


Figure 2 Zinc oxide nanoparticle reduces the invasion and migration of gastric cancer cells. SGC7901 and BGC823 cells were treated with zinc oxide nanoparticle (5 μg/mL, ZnO-NP) or an equal volume of saline. A and B: Cell migration and invasion were determined by transwell assays; C and D: Migration and invasion were examined by wound healing assays. The wound healing proportion is shown. Data are presented as the mean ± SD. Statistically significant differences are indicated: $^{b}P < 0.01$.

therapy resistance of GC cells is unclear. In this study, we found that ZnO-NP attenuated the chemoresistance of GC cells by inhibiting autophagy.



	Mock	ZnO-NP
IC50 (μmol/L)	10.15	4.55

Figure 3 Zinc oxide nanoparticle attenuates the chemotherapy drug resistance of gastric cancer cells. A: SGC7901/cisplatin (DDP) cells were treated with DDP at the indicated doses and treated with zinc oxide nanoparticle (ZnO-NP, 5 μg/mL) or an equal volume of saline. Cell viability was measured by the MTT assay; B and C: SGC7901/DDP cells were treated with DDP or co-treated with DDP and ZnO-NP (5 μg/mL). Cell apoptosis was assessed by flow cytometry. Data are presented as the mean ± SD. Statistically significant differences are indicated: ns, no significance, $^{a}P < 0.05$. FITC: Fluorescein isothiocyanate.

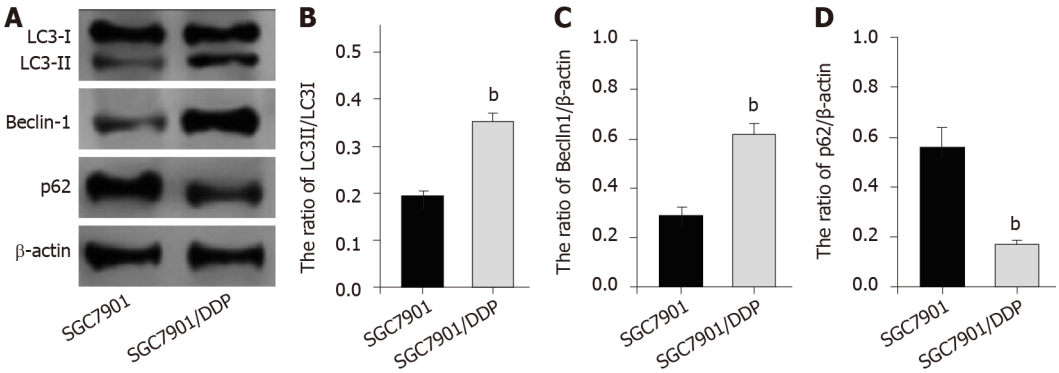


Figure 4 Autophagy is increased in chemotherapy-resistant gastric cancer cells. A: Expression of light chain 3B-II (LC3B-II), LC3B-I, Beclin-1, p62, and β-actin was measured by Western blot analysis in SGC7901 and SGC7901/cisplatin (DDP) cells; B: Ratio of LC3II/LC3I; C: Ratio of Beclin-1/β-actin; D: Ratio of p62/β-actin. The results of Western blot analysis were quantified by ImageJ software. Data are presented as the mean ± SD. Statistically significant differences are indicated: $^{b}P < 0.01$.

Previous studies have identified the cancer-inhibitory effect of ZnO-NP. ZnO-NP promotes proteotoxic and oxidative stress and induces the apoptosis of ovarian cancer cells in a p53-mutation-dependent manner[27]. ZnO-NP enhances the cell death of multiple human myelomas by regulating reactive oxygen species and cytochrome c/apoptotic protease activating factor 1/caspase-9 signaling[28]. ZnO-NP increases the apoptosis of human ovarian cancer cells[29]. Frizzled-7-targeted delivery of ZnO-NP

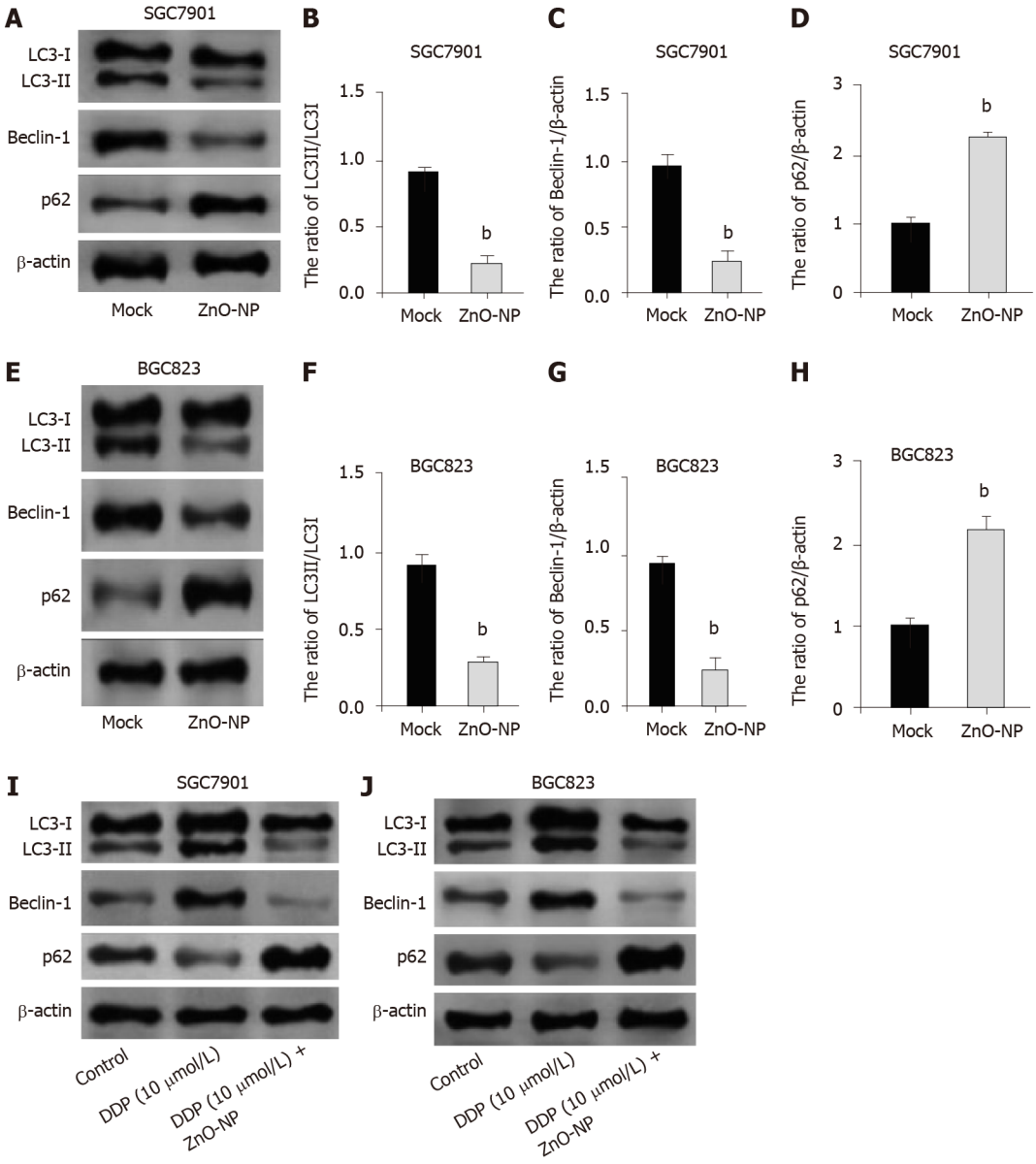


Figure 5 Zinc oxide nanoparticle inhibits autophagy in gastric cancer cells. A-H: SGC7901 and BGC823 cells were treated with zinc oxide nanoparticle (ZnO-NP, 5 μg/mL) or an equal volume of saline. The expression of light chain 3B-II (LC3B-II), LC3B-I, Beclin-1, p62, and β-actin was measured by Western blot analysis. The results of Western blot analysis were quantified by ImageJ software; I and J: SGC7901 and BGC823 cells were treated with cisplatin (DDP, 10 μmol/L) or co-treated with DDP (10 μmol/L) and ZnO-NP (5 μg/mL). The expression of LC3B-II, LC3B-I, Beclin-1, p62, and β-actin was analyzed by Western blot analysis. Data are presented as the mean ± SD. Statistically significant differences are indicated: $^{b}P < 0.01$.

induces inhibitory effects on the drug resistance of breast cancer cells[30]. Moreover, iron NPs reverse the chemotherapy resistance of GC cells[31]. In this study, we found that ZnO-NP inhibited proliferation, migration, and invasion and induced apoptosis of GC cells. Meanwhile, ZnO-NP attenuated the DDP resistance of GC cells. Moreover, ZnO-NP was able to repress the tumor growth of chemoresistance GC cells *in vivo*. Our findings indicate the innovative effect of ZnO-NP on the chemotherapy drug resistance of GC cells, demonstrating critical evidence of metal oxide NPs in the regulation of cancer development.

Furthermore, previous studies have identified that autophagy is clearly correlated with chemotherapy drug resistance and the development of GC, and targeting autophagy is involved in the modulation of chemoresistance GC cells. Long noncoding RNA MALAT1 modulates autophagy-related chemoresistance by targeting miR-23b-3p in GC[14]. Autophagy contributes to the chemoresistance of GC stem cells by regulating Notch signaling[32]. Tripartite motif containing 14 induces autophagy and chemotherapy resistance of GC cells *via* modulating adenosine monophosphate-activated protein kinase/mechanistic target of rapamycin (mTOR) signaling[33]. Cluster of differentiation 133 (CD133) inhibition reduces DDP resistance by repressing

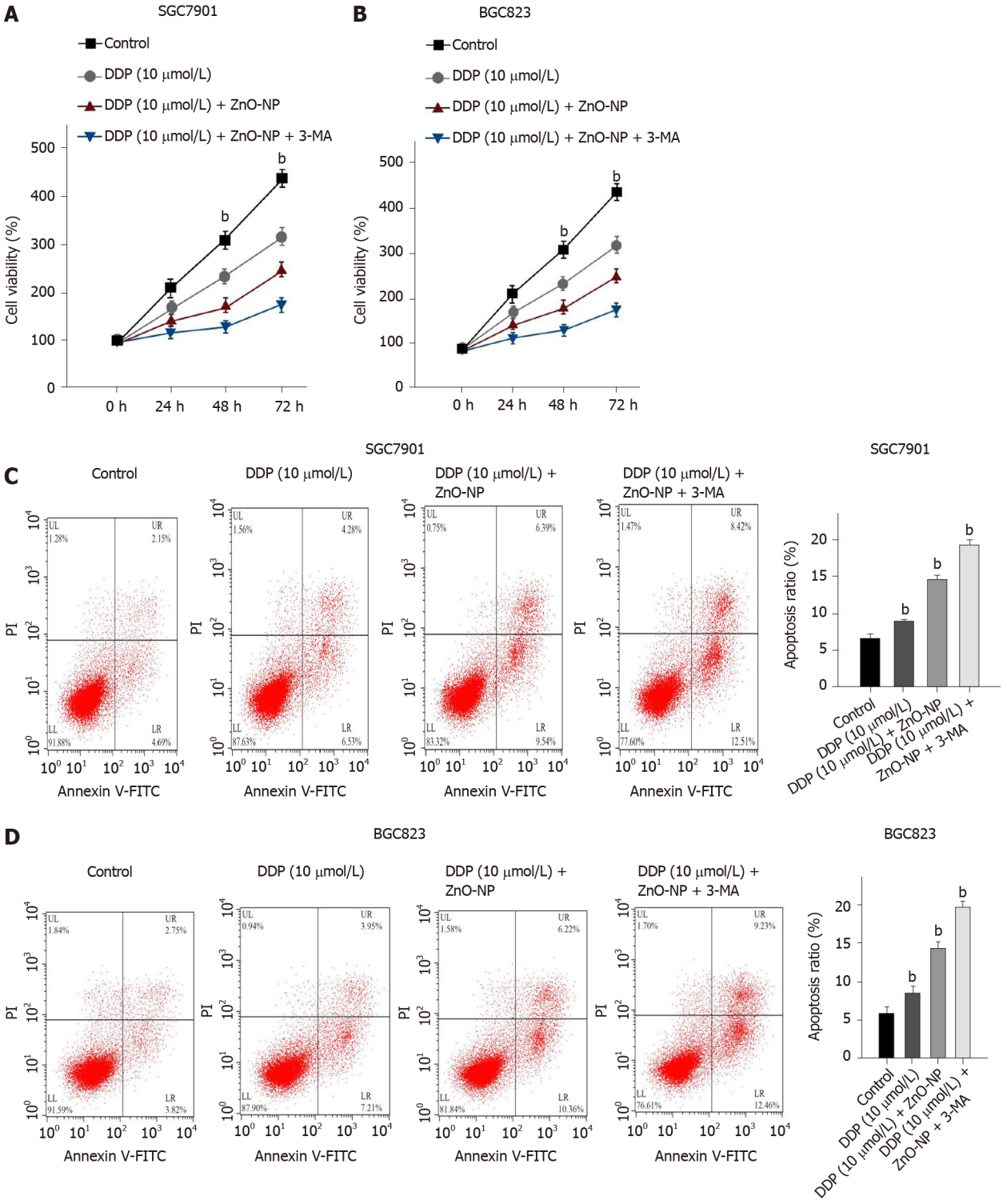


Figure 6 Zinc oxide nanoparticle attenuates chemotherapy drug resistance by inhibiting the autophagy of gastric cancer cells. SGC7901 and BGC823 cells were treated with cisplatin (DDP), DDP and zinc oxide nanoparticle (ZnO-NP, 5 μg/mL), co-treated with DDP, ZnO-NP (5 μg/mL) and 3-methyladenine (3-MA, 5 mmol/L). A and B: Cell viability was determined by the MTT assay; C and D: Cell apoptosis was analyzed by flow cytometry. Data are presented as the mean ± SD. Statistically significant differences are indicated: $^{b}P < 0.01$. FITC: Fluorescein isothiocyanate.

autophagy and phosphatidylinositol 3-kinase/AKT/mTOR signaling in CD133-positive GC cells[34].

CDGSH iron sulfur domain 2 improves the chemosensitivity of GC cells by enhancing 5-FU-promoted apoptosis and inhibiting autophagy through AKT/mTOR signaling[35]. In this study, our data showed that autophagy was increased in chemotherapy-resistant GC cells. Treatment with DDP induced autophagy in the cells, which was reversed with treatment of ZnO-NP. ZnO-NP attenuated chemotherapy drug resistance by inhibiting the autophagy of GC cells[36]. These data reveal an

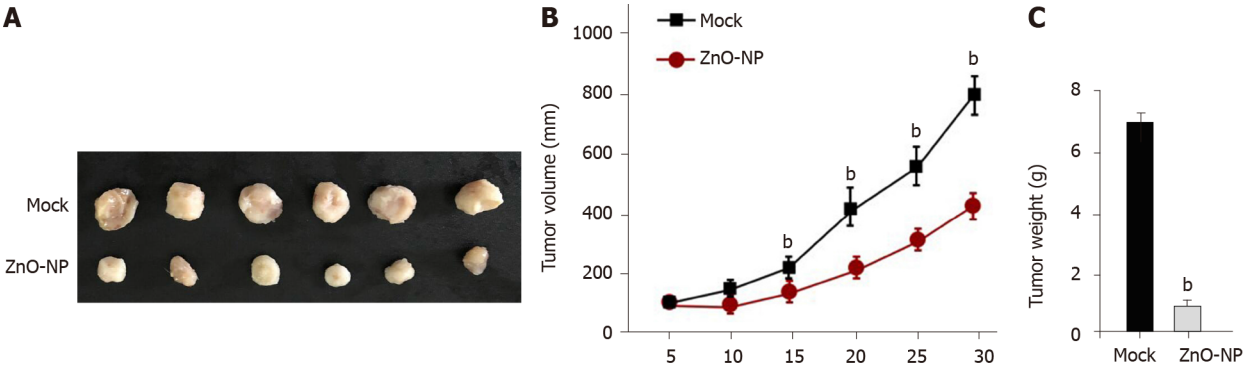


Figure 7 Zinc oxide nanoparticle reduces the tumor growth of chemoresistant gastric cancer cells *in vivo*. The impact of zinc oxide nanoparticle (ZnO-NP) on tumor growth of cisplatin (DDP)-resistant gastric cells *in vivo* was analyzed by the nude mice tumorigenicity assay (n = 5). SGC7901/DDP cells were treated with ZnO-NP (5 μg/mL) or an equal volume of saline. A: Representative images of dissected tumors from nude mice are shown; B: The average tumor volume was calculated; C: The average tumor weight was calculated. Data are presented as the mean ± SD. Statistic significant differences are indicated: $^{b}P < 0.01$.

unreported mechanism involving autophagy underlying ZnO-NP-induced anti-tumor function and inhibition of chemotherapy drug resistance of GC cells, demonstrating the association of ZnO-NP with autophagy in cancer cells.

CONCLUSION

In summary, we conclude that ZnO-NP reduces the chemoresistance of GC cells by inhibiting autophagy. Our findings provide innovative insights into the scenario in which ZnO-NP mediates the chemotherapy resistance of GC. ZnO-NP may serve as a potential therapeutic candidate for GC treatment. The potential role of ZnO-NP in the clinical treatment of GC needs to be clarified in future investigations.

ARTICLE HIGHLIGHTS

Research background

Gastric cancer (GC) is a common cancer and results in a high rate of tumor-related mortality. Cisplatin (DDP)-based chemotherapy is the first-line treatment of GC, but chemoresistance remains a severe clinical problem. Zinc oxide nanoparticle (ZnO-NP) has been identified as a promising anti-cancer agent, but its role in GC development is still unclear.

Research motivation

To identify the role of ZnO-NP in the regulation of GC progression.

Research objectives

This study explored the effect of ZnO-NP on chemotherapy resistance during GC progression.

Research methods

ZnO-NP was synthesized, and the effect and underlying mechanism on the malignant progression and chemotherapy resistance of GC cells were assessed by tumorigenicity in nude mice and evaluated by Western blotting, flow cytometry analysis, wound healing assays, transwell assays, colony formation assays, and MTT assays in GC cells and DDP-resistant GC cells.

Research results

ZnO-NP inhibited proliferation, migration, and invasion and induced apoptosis of GC cells. Meanwhile, ZnO-NP significantly reduced the IC_{50} value of DDP for the inhibition of cell proliferation of DDP-resistant SGC7901/DDP cell lines. Autophagy was increased in the chemotherapy-resistant GC cells, as demonstrated by elevated LC3II/LC3I and Beclin-1 expression and repressed p62 expression in the SGC7901/

DDP compared with that in SGC7901 cells. Mechanically, ZnO-NP inhibited autophagy in GC cells, and treatment with DDP induced autophagy in the cells, which was reversed by ZnO-NP. Functionally, ZnO-NP attenuated the tumor growth of chemoresistant GC cells *in vivo*.

Research conclusions

ZnO-NP alleviates the chemoresistance of GC cells by inhibiting autophagy. Our findings provide innovative insights into the scenario in which ZnO-NP mediates chemotherapy resistance in GC.

Research perspectives

ZnO-NP may serve as a potential therapeutic candidate for GC treatment. The potential role of ZnO-NP in the clinical treatment of GC needs to be clarified in future investigations.

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ORIGINAL ARTICLE

Basic Study

PPARGC1A rs8192678 G>A polymorphism affects the severity of hepatic histological features and nonalcoholic steatohepatitis in patients with nonalcoholic fatty liver disease

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Author contributions: Zhang RN and Shen F contributed equally to this work; Zhang RN, Pan Q, Shen F, Chen GY, and Cao HX enrolled the cohorts and collected blood samples; Zhang RN, Pan Q, and Shen F performed genotyping; Zhang RN, Pan Q interpreted the data and were involved in the manuscript preparation; Fan JG designed and supervised the study; Zhang RN and Fan JG wrote the manuscript.

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Institutional review board statement: This study was

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Abstract

BACKGROUND

The association between PPARGC1A rs8192678 and nonalcoholic fatty liver disease (NAFLD) requires further confirmation. In addition, it is still unknown whether PPARGC1A rs8192678 is associated with hepatic histological features in NAFLD in the Chinese population.

AIM

To investigate the interaction between PPARGC1A rs8192678 and nonalcoholic steatohepatitis (NASH), and whether this polymorphism is associated with hepatic histological features.

METHODS

Fifty-nine patients with liver biopsy-proven NAFLD and 93 healthy controls were recruited to a cohort representing the Chinese Han population. The SAF (steatosis, activity, and fibrosis) scoring system was used for hepatic histopathological evaluation. The polymorphisms of PPARGC1A rs8192678 and patatin-like phospholipase domain-containing protein 3 (PNPLA3) rs738409 were genotyped. The intrahepatic mRNA expression of PPARGC1A was evaluated by real-time polymerase chain reaction.

approved by the institutional review board of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine.

Conflict-of-interest statement: The authors declare that there is no conflict of interest related to this study.

Data sharing statement: No additional data are available.

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RESULTS

Thirty-seven patients with NAFLD had NASH, of which 12 were nonobese. The PPARGC1A rs8192678 risk A allele (carrying GA and AA genotypes) had the lowest *P* value in the dominant model; the odds ratio (OR) for NAFLD was 2.321 [95% confidence interval (CI): 1.121-4.806]. After adjusting for age, sex, and the PNPLA3 rs738409 risk G allele, the PPARGC1A rs8192678 A allele was a risk factor for NAFLD (OR 2.202, 95%CI: 1.030-4.705, $P = 0.042$). The genetic analysis showed that patients with NAFLD, moderate-to-severe steatosis (S2-3), and Activity 2-4 (A ≥ 2) were more likely to carry A in PPARGC1A rs8192678 (OR 5.000, 95%CI: 1.343-18.620, $P = 0.012$; and OR 4.071, 95%CI: 1.076-15.402, $P = 0.031$). The multivariate logistic regression analysis showed that PPARGC1A rs8192678 risk A allele was also independently associated with S2-3, A ≥ 2, and NASH (OR 6.190, 95%CI: 1.508-25.410, $P = 0.011$; OR 4.506, 95%CI 1.070-18.978, $P = 0.040$; and OR 6.337, 95%CI: 1.135-35.392, $P = 0.035$, respectively) after adjusting for age, sex, body mass index, and PNPLA3 rs738409 risk G allele. The results also showed that this polymorphism was associated with nonobese NASH (OR 22.000, 95%CI: 1.540-314.292, $P = 0.021$). The intrahepatic expression of PPARGC1A mRNA was significantly lower in the group of patients who carried the risk A allele ($P = 0.014$).

CONCLUSION

The PPARGC1A rs8192678 risk A allele is associated with NAFLD, and with S2-3, A ≥ 2 and NASH in NAFLD patients, independent of PNPLA3 rs738409, and may be associated with nonobese NASH.

Key Words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; PPARGC1A rs8192678 polymorphism; Steatosis; Activity

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Core Tip: The PPARGC1A rs8192678 A allele was found to be a risk factor for liver biopsy-proven nonalcoholic fatty liver disease (NAFLD) after adjusting for age, sex, and patatin-like phospholipase domain-containing protein 3 rs738409 polymorphism. The multivariate logistic regression analysis showed that the PPARGC1A rs8192678 risk A allele was also independently associated with the severity of hepatic histological features (S2-3 and A ≥ 2) and nonalcoholic steatohepatitis (NASH), and might also be associated with nonobese NASH, indicating that the PPARGC1A rs8192678 risk A allele was associated with NAFLD in the Chinese Han adult population. Also, the intrahepatic expression of PPARGC1A mRNA was significantly lower in the patients who carried the risk A allele, implying that it might be a genetic contribution to the pathogenesis of NAFLD.

Citation: Zhang RN, Shen F, Pan Q, Cao HX, Chen GY, Fan JG. PPARGC1A rs8192678 G>A polymorphism affects the severity of hepatic histological features and nonalcoholic steatohepatitis in patients with nonalcoholic fatty liver disease. *World J Gastroenterol* 2021; 27(25): 3863-3876
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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) has become one of the most common forms of chronic liver diseases worldwide. The overall global prevalence of NAFLD is about 25%. The clinical spectrum of NAFLD ranges from nonalcoholic fatty liver (NAFL), nonalcoholic steatohepatitis (NASH), liver fibrosis and cirrhosis, and finally hepatocellular carcinoma. NAFLD also has high risk and is associated with multisystem diseases, such as obesity, type 2 diabetes mellitus (T2DM), cardiovascular diseases, chronic kidney disease, and malignancy[1,2].

The pathogenesis of NAFLD is multifactorial and strongly associated with obesity, insulin resistance, T2DM, and metabolic syndrome. Although NAFLD has a high prevalence in obese patients, nonobese patients and even lean patients, were recently found to have NAFLD as well. The anthropometric data on body mass index (BMI) was used to define nonobese or lean. The BMI values of 25 and 30 kg/m^2 are the thresholds to define overweight and obese participants, respectively, as recommended by the World Health Organization. In Asia, BMI < 25 kg/m^2 is defined as nonobese, whereas BMI < 23 kg/m^2 is defined as lean[3]. A meta-analysis was carried out to estimate the overall prevalence of NAFLD in nonobese and lean populations (15.7% and 10.2%, respectively)[4].

The differences in prevalence, clinical profile, hepatic histology severity, and outcomes of NAFLD among different ethnic groups suggested that both environmental and genetic factors influenced susceptibility to NAFLD in individuals[5]. Genetic factors have been implicated in the occurrence and development of NAFLD; the gene of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), encoded by PPARGC1A, is also a susceptibility candidate gene for NAFLD[6-8]. PGC-1α is a transcriptional coactivator with a crucial impact on multiple aspects of cellular energy metabolism including mitochondrial biogenesis and cellular respiration, regulation of adaptive thermogenesis, adipocyte cell development, and lipid and glucose metabolism. A number of genetic variants have been identified in PPARGC1A, and the Gly482Ser polymorphism (a G-to-A transition that predicted a glycine (G) to serine (S) substitution at amino acid position 482 in exon 8, rs8192678) is the most common variant, which was associated with T2DM, obesity, and hypertension in several studies[9-13]. However, the results were controversial in the Chinese Han adult population, which require further confirmation[14,15]. Whether this polymorphism is associated with the severity of hepatic histological features [steatosis, activity (hepatocyte ballooning and lobular inflammation), and fibrosis] and with NASH or nonobese NASH needs further investigation.

The present study aimed to determine the relationship between polymorphism rs8192678 of *PPARGC1A* gene and the risk of NAFLD, especially in NASH, in a Chinese Han population, and to investigate the association between PPARGC1A rs8192678 G>A polymorphism and the severity of hepatic histological features.

MATERIALS AND METHODS

Study populations

A total of 152 unrelated adult participants (18-70 years old) were recruited between March 2012 and March 2013. Patients with NAFLD were enrolled from Xinhua Hospital, Shanghai, China. All participants were Han Chinese in origin. Each patient had undergone an ultrasound-guided percutaneous liver biopsy and met the diagnostic criteria for NAFLD. The liver stiffness measurement (LSM) and controlled attenuation parameter (CAP) values were tested using FibroScan 502 (Echosens, Paris, France) by previously described methods.

The exclusion criteria were as follows: (1) excessive alcohol consumption (> 30 g/d for men and > 20 g/d for women); other diseases that led to fatty liver, such as chronic hepatitis C, drug-induced liver injury, Wilson's disease, total parenteral nutrition, and autoimmune hepatitis; (2) previous liver transplantation; (3) other end-stage diseases or malignancies; and (4) contraindications to FibroScan examination (*e.g.*, ascites, implanted pacemakers, nonhealing wounds in the upper-right quadrant of the abdomen, and pregnancy) or unreliable measurement of LSM and CAP values.

All control participants were confirmed to be free of liver diseases by both B-mode ultrasound and FibroScan 502 examination (CAP ≤ 240 dB/m and LSM values < 7.0 kPa)[16]; they all had normal liver function test results and lacked evidence of etiologies of liver injury. The study protocol was approved by the Ethics Committees of Xinhua Hospital, and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

Clinical and laboratory evaluation

Demographic information, such as age and sex, was collected. Venous blood samples were obtained from the participants after overnight fasting (12 h) to measure the levels of alanine transaminase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), total bilirubin, directed bilirubin, fasting blood glucose (FBG), total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and uric acid (UA). All laboratory biochemical

parameters were measured in a conventional automated analyzer (Hitachi 7600, Tokyo, Japan).

Hepatic histopathologic assessment

Liver biopsy specimens were fixed with 10% buffered formalin, embedded in paraffin, sliced, and stained with hematoxylin & eosin (HE), reticulin, and Masson trichrome. A minimum 15-mm biopsy specimen or the presence of at least 10 complete portal tracts was required. For each liver biopsy, the steatosis, activity, and fibrosis (SAF) score summarizing the main histological lesions was defined. The SAF score was designed by the fatty liver inhibition of progression Pathology Consortium. The steatosis (0-3) was categorized as follows: S0: < 5%; S1: 5%-33%; S2: 34%-66 %; and S3: ≥ 67%. The grade of hepatic inflammatory activity (A, from 0 to 4) was calculated by adding together the grades for hepatocyte ballooning (0-2) and lobular inflammation (0-2). Liver fibrosis was staged as follows: F0, none; F1, perisinusoidal or portal fibrosis; F2, perisinusoidal and periportal fibrosis without bridging; F3, bridging fibrosis; and F4, cirrhosis. A case presenting with at least grade 1 of each of the three features (steatosis, ballooning, and lobular inflammation) was classified as NASH; other cases were diagnosed as NAFL.

DNA preparation and single-nucleotide polymorphism genotyping

Polymorphism genotyping of genomic DNA was prepared from each blood sample using the QiAamp DNA Mini Kit (Qiagen, Hilden, Germany). The custom Ion AmpliSeq panel (Life Technologies, MA, United States) of PPARGC1A and patatin-like phospholipase domain-containing protein 3 (PNPLA3) was designed. The polymerase chain reaction (PCR) of the template was performed using the Ion OneTouch 2 System (Life Technologies, MA, United States) following the manufacturer's instructions. PPARGC1A and PNPLA3 variants were genotyped by DNA sequencing using the Ion 318 Chip (Life Technologies, United States) following the Ion PGM 200 Sequencing kit protocol. Negative controls were introduced for each run to ensure genotyping quality. Samples giving discordant results were reanalyzed.

Real-time PCR for measuring PPARGCA1 mRNA expression

Total RNA was isolated from samples of liver biopsy specimens using the TRIzol reagent (Takara, Dalian, China) and reverse transcribed using the PrimeScript RT Reagent Kit (Takara, Dalian, China). The mRNA expression of PPARGC1A and β-actin was measured by real-time PCR (RT-PCR) using the SYBR Premix Ex Taq Kit (Takara, Dalian, China) and an ABI 7500 RT-PCR System (Applied Biosystems, United States). Target mRNA levels were normalized to β-actin expression levels. The following primers were used: PPARGC1A (F: 5'-GAGTGACATCGAGTGTGCTG-3'; R: 5'-GGGCAATCCGTCTTCATCCA-3'); β-actin (F: 5'-TCCTTCCTGGGCATGGAGT-3'; R: 5'-CAGGAGGAGCAATGATCTTGAT-3'). A duplicate of this experiment was performed for the same reaction. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of each gene. Thermal cycling was performed as follows: Initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 34 s.

Statistical analysis

Continuous variables were expressed as mean ± SD, median and interquartile range for those with a skewed distribution and categorical variables as the frequency or percentage. The *t* test and chi-squared test were used where appropriate. Multiple logistic regression models were used to assess the factors independently associated with the severity of histological parameters. In the dominant model, the frequencies of the participants with the allele 1 (allele 1 homozygote and heterozygote) were compared with the participants homozygous for the allele 2 using a 2 × 2 contingency table. The odds ratio (OR) and the 95% confidence interval (CI) were calculated using Woolf's method. In all analyses, GA and AA for PPARGC1A rs8192678 was considered when choosing a dominant model of inheritance. Analyses were performed using SPSS 23.0 (SPSS Inc., IL, United States). A two-sided $P < 0.05$ indicated a statistically significant difference.

RESULTS

Anthropometric and clinical data

The clinical characteristics of 152 participants (59 patients with NAFLD and 93

controls) are listed in Table 1. Age, BMI, ALT, GGT, TC, TG, LDL-C, UA, CAP, and LSM values were significantly higher in the NAFLD group. No differences in sex, ALP, HDL-C, and FBG were observed between the two groups. Among the patients with NAFLD, 37 (62.71%) had NASH, and 22 (37.29%) had NAFL. Furthermore, 69.49% (41/59) were obese, and 30.51% (18/59) were nonobese. Patients with NAFLD also showed a significantly higher frequency of hypertension and T2DM. The ALT, GGT, CAP, and LSM levels were significantly higher in patients with NASH compared to patients with NAFL, with no significant difference in the frequency of obesity, hypertension, and T2DM (Tables 1 and 2).

PPARGC1A rs8192678 polymorphism genotypes and allele frequencies and the risk of NAFLD between different groups

The percentage of PPARGC1A rs8192678 GA genotype was significantly higher in the NAFLD group than in the control group (62.71% *vs* 39.78 %), suggesting that carriers of this genotype had a significantly increased risk of NAFLD (OR 2.786, 95%CI: 1.300-5.962, P = 0.008) (Table 3). The carriers of the homozygous AA genotype had no significantly increased risk of NAFLD compared to participants with GG homozygous alleles (P = 0.609). The close association was further revealed in the dominant model (combined GA and AA genotypes, GA + AA *vs* GG, OR 2.321, 95%CI: 1.121-4.806, P = 0.022) (Tables 3 and 4).

The percentage of the PNPLA3 rs738409 GG genotype was also significantly higher in patients with NAFLD than in controls (33.90% *vs* 16.13%, P = 0.004). The OR of NAFLD was 3.889-fold (95%CI: 1.534-9.926, P = 0.004) higher in participants with the GG genotype than in those with the CC genotype; and the participants carrying G allele also had a high risk of NAFLD (GG + GC *vs* CC, OR 2.364, 95%CI: 1.105-5.055, P = 0.025) (Table 3). The multiple logistic regression analysis revealed that the PPARGC1A rs8192678 A allele was a risk factor for NAFLD (OR 2.202, 95%CI: 1.030-4.705, P = 0.042) independent of age, sex, and PNPLA3 rs738409 polymorphism (Table 5).

Association between PPARGC1A rs8192678 polymorphism and hepatic histological features

The hepatic histological features, including steatosis, activity (lobular inflammation and ballooning), and fibrosis, were assessed in patients with NAFLD. The frequencies of PPARGC1A rs8192678 GA + AA genotypes were remarkably higher in patients with moderate-to-severe steatosis (S2-3) and activity 2-4 (A ≥ 2) compared with those with mild steatosis (S1) and activity 0-1 (A < 2). Also, the carriers with risk A allele (carrying GA and AA genotypes) had a significantly increased risk in NAFLD patients with S2-3 and A ≥ 2 (OR 5.000, 95%CI: 1.343-18.620, P = 0.012 and OR 4.071, 95%CI: 1.076-15.402, P = 0.031). The multivariate analysis showed that PPARGC1A rs8192678 A allele was also significantly associated with S2-3 and A ≥ 2 (OR 6.190, 95%CI: 1.508-25.410, P = 0.011 and OR 4.506, 95%CI: 1.070-18.978, P = 0.040) after adjusting for age, sex, BMI, and PNPLA3 rs738409 risk G allele. However, no significant differences were found in significant fibrosis 2-4 (F2-4) and advanced fibrosis 3-4 (F3-4) compared with F0-1 and F0-2 (P = 0.186 and P = 0.426) (Figure 1A-E and Table 6).

The patients with NASH carrying PPARGC1A rs8192678 GA + AA genotypes had an OR of 4.431 (95%CI: 1.245-15.763, P = 0.017) when compared with NAFL (Table 4). The multivariate analysis also showed that PPARGC1A rs8192678 risk A allele was independently associated with NASH after adjusting for age, sex, BMI, and PNPLA3 rs738409 risk G allele in patients with NAFLD (OR 6.337, 95%CI: 1.135-35.392, P = 0.035) (Table 6).

PPARGC1A rs8192678 genotype is associated with nonobese NASH patients

The GA + AA genotypes were not associated with obese NAFLD, obese NASH, and obese NAFL in the dominant model compared with nonobese NAFLD, nonobese NASH, and nonobese NAFL, respectively. Genetic analysis also showed no significant difference in the patients carrying A allele in PPARGC1A rs8192678 between obese NASH and obese NAFL groups (Table 4).

The remaining anthropometric values, biochemical test values, and PNPLA3 polymorphism did not show any significant differences between the two groups, apart from a trend in a higher level of GGT (P = 0.068) and CAP (P = 0.056) in nonobese NASH. However, genetic analysis showed that the proportion of PPARGC1A rs8192678 GA + AA genotypes was significantly higher in the patients with nonobese NASH compared with patients with nonobese NAFL, which was significantly associated with nonobese NASH (OR 22.000, 95%CI: 1.540-341.292, P = 0.021) (Tables 4

Table 1 Clinical characteristics of patients with nonalcoholic fatty liver disease and controls

Variables	NAFLD (*n* = 59)	Controls (*n* = 93)	*P* value
Sex (M/F)	43/16	59/34	0.227
Age (year)	38.20 ± 13.78	42.20 ± 11.05	0.039
BMI (kg/m^2)	27.31 ± 3.31	23.06 ± 2.74	< 0.0001
ALT (U/L)	70.56 ± 47.6	24.98 ± 16.07	< 0.0001
GGT (U/L)	72.75 ± 55.22	25.94 ± 18.17	< 0.0001
ALP (U/L)	97.07 ± 44.73	96.50 ± 32.54	0.347
TC (mmol/L)	4.86 ± 0.86	4.39 ± 0.89	0.001
TG (mmol/L)	1.99 ± 1.25	1.14 ± 0.74	< 0.0001
HDL-C (mmol/L)	1.20 ± 0.28	1.23 ± 0.28	0.177
LDL-C (mmol/L)	2.83 ± 0.7	2.24 ± 0.50	< 0.0001
FBG (mmol/L)	5.93 ± 2.62	5.31 ± 0.79	0.941
UA (μmol/L)	361.32 ± 105.78	274.25 ± 76.53	< 0.0001
CAP (dB/m)	313 (277-351)	212 (172-232)	< 0.0001
LSM (kPa)	7.7 (5.6-12)	5.25 (4.1-7.0)	< 0.0001
Hypertension, *n* (%)	22 (37.29%)	15 (16.13%)	0.003
T2DM, *n* (%)	7 (11.86%)	1 (1.08%)	0.005
Obesity, *n* (%)	41 (69.49%)	19 (20.43%)	< 0.0001
Steatosis (S, *n*)			
S1/S2/S3	25/25/9	/	/
Activity (A, *n*)			
A0/A1/A2/A3/A4	1/12/16/22/8	/	/
Fibrosis (F, *n*)			
F0/F1/F2/F3/F4	11/29/8/7/4	/	/

The data are expressed as mean ± SD, or as *n* (%) for normally distributed variables and as median (interquartile range) when distribution of the variable was skewed. NAFLD: Nonalcoholic fatty liver disease; BMI: Body mass index; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyl transpeptidase; TC: Total cholesterol; TG: Triglycerides; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; FBG: Fasting blood glucose; UA: Uric acid; LSM: Liver stiffness measurement; CAP: Controlled attenuation parameter; T2DM: Type 2 diabetes mellitus.

and 7).

mRNA expression of PPARGC1A in the liver of patients with NAFLD

The expression of PPARGC1A mRNA in the liver biopsy specimens of patients with NAFLD was evaluated by RT-PCR. The results obtained by RT-PCR in the GA or AA group (*n* = 5) were compared with the results in the GG group (*n* = 5) at PPARGC1A rs8192678. As shown in Figure 1F, the expression of PPARGC1A mRNA transcripts in the liver was significantly lower in the GA or AA group than in the GG group (*P* = 0.014).

DISCUSSION

Genetic factors may be important in the development of NAFLD, in either obese or nonobese patients. The present study further indicated that PPARGC1A rs8192678 risk A allele was associated with liver biopsy-proven NAFLD susceptibility in a Chinese Han adult population, and it was especially associated with NASH compared with NAFL. The PPARGC1A rs8192678 risk A allele was also associated with nonobese NASH in patients with nonobese NAFLD. In addition, this polymorphism was

Table 2 Clinical characteristics of patients with nonalcoholic steatohepatitis and nonalcoholic fatty liver among patients with nonalcoholic fatty liver disease

Variable	NAFL (*n* = 22)	NASH (*n* = 37)	*P* value
Sex (M/F)	17/5	26/11	0.559
Age (year)	37.23 ± 13.64	38.78 ± 14.01	0.605
BMI (kg/m^2)	27.07 ± 3.69	27.38 ± 3.17	0.675
ALP (U/L)	102.96 ± 52.34	93.56 ± 39.91	0.982
GGT (U/L)	38.00 (23.80-69.03)	69.70 (39.00-85.50)	0.023
ALT (U/L)	46.00 (30.33-64.95)	71.40 (46.9-111.70)	0.006
TC (mmol/L)	5.03 ± 1.04	4.76 ± 0.72	0.801
TG (mmol/L)	1.91 ± 0.74	2.04 ± 1.48	0.532
HDL-C (mmol/L)	1.21 ± 0.28	1.19 ± 0.28	0.903
LDL-C (mmol/L)	2.97 ± 0.91	2.72 ± 0.49	0.444
FBG (mmol/L)	6.43 ± 3.59	5.62 ± 1.77	0.318
UA (μmol/L)	360.91 ± 107.80	361.62 ± 106.22	0.482
CAP (dB/m)	295.50 (249.25-345.00)	333.00 (296.25-356.25)	0.038
LSM (kPa)	6.20 (4.55-9.95)	9.60 (6.50-12.85)	0.008
Obesity, *n* (%)	16 (72.73)	25 (60.97)	0.677
Hypertension, *n* (%)	8 (36.36)	14 (37.84)	0.910
T2DM, *n* (%)	3 (13.64)	4 (10.81)	0.746

The data are expressed as mean ± SD, or as *n* (%) for normally distributed variables and as median (interquartile range) when distribution of the variable was skewed. NAFL: Nonalcoholic fatty liver; NASH: Nonalcoholic steatohepatitis; BMI: Body mass index; ALP: Alkaline phosphatase; GGT: γ-glutamyl transpeptidase; ALT: Alanine aminotransferase; TC: Total cholesterol; TG: Triglycerides; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; FBG: Fasting blood glucose; UA: Uric acid; LSM: Liver stiffness measurement; CAP: Controlled attenuation parameter; T2DM: Type 2 diabetes mellitus.

Table 3 Genotype distribution of PPARGC1A rs8192678 and patatin-like phospholipase domain-containing protein 3 rs738409 in patients with nonalcoholic fatty liver disease and controls

Genotype	NAFLD (*n* = 59)	Controls (*n* = 93)	χ^2	*P* value	OR (95%CI)
PPARGC1A rs8192678					
GG	14 (23.73%)	39 (41.94%)			
GA	37 (62.71%)	37 (39.78%)	7.148	0.008	2.786 (1.300-5.962)
AA	8 (13.56%)	17 (18.28%)	0.262	0.609	1.311 (0.464-3.704)
GA+AA	45 (76.27%)	54 (58.06%)	5.269	0.022	2.321 (1.121-4.806)
PNPLA3 rs738409					
CC	12 (20.34%)	35 (37.63%)			
CG	27 (45.76%)	43 (46.24%)	2.152	0.142	1.831 (0.812-4.131)
GG	20 (33.90%)	15 (16.13%)	8.423	0.004	3.889 (1.524-9.926)
CG + GG	47 (79.56%)	58 (62.37%)	5.055	0.025	2.364 (1.105-5.055)

OR: Odds ratio; CI: Confidence interval; PNPLA3: Patatin-like phospholipase domain-containing protein 3; NAFLD: Nonalcoholic fatty liver disease.

associated with moderate-to-severe steatosis and higher histological activity, but not with significant and advanced fibrosis.

PPARGC1A/PGC-1α is a coactivator for a number of transcription factors, including the peroxisome proliferator-activated receptor. PGC-1α is a critical regulator

Table 4 Association tests of PPARGC1A rs8192678 polymorphism between different groups

PPARGC1A rs8192678	Case			Control			Dominant model		
	GG	GA	AA	GG	GA	AA	χ^2	*P* value	OR (95%CI)
NAFLD *vs* Control	14	37	8	39	37	17	5.269	0.022	2.321 (1.121-4.806)
NASH *vs* Control	5	28	4	39	37	17	9.550	0.002	4.622 (1.653-12.929)
NASH *vs* NAFL	5	28	4	9	9	4	5.721	0.017	4.431 (1.245-15.763)
Obese *vs* nonobese participants	17	33	10	36	41	15	1.864	0.172	1.626 (0.807-3.276)
Obese NAFLD *vs* nonobese NAFLD	9	27	5	5	10	3	0.235	0.628	1.368 (0.384-4.865)
Obese NASH *vs* nonobese NASH	4	19	2	1	9	2	0.408	0.348	0.477 (0.047-4.806)
Obese NAFL *vs* nonobese NAFL	5	8	3	4	1	1	2.264	0.132	4.400 (0.596-32.501)
Obese NASH *vs* obese NAFL	4	19	2	5	8	3	1.324	0.250	2.386 (0.531-10.734)
Nonobese NASH *vs* nonobese NAFL	1	9	2	4	1	1	6.785	0.021	22.000 (1.540-314.292)

NAFL: Nonalcoholic fatty liver; NASH: Nonalcoholic steatohepatitis; NAFLD: Nonalcoholic fatty liver disease; OR: Odds ratio; CI: Confidence interval.

Table 5 Multiple logistic regression analysis of predictive factors for nonalcoholic fatty liver disease

Variables	B	S.E.	Wald	OR (95%CI)	*P* value
Age (year)	-0.031	0.015	4.236	0.970 (0.942-0.999)	0.040
Sex (Male)	-0.233	0.385	0.365	0.793 (0.373-1.686)	0.546
PPARGC1A rs8192678 A allele	0.789	0.388	4.148	2.202 (1.030-4.705)	0.042
PNPLA3 rs738409 G allele	0.857	0.403	4.524	2.356 (1.070-5.191)	0.033

OR: Odds ratio; CI: Confidence interval; PNPLA3: Patatin-like phospholipase domain-containing protein 3.

Table 6 Association of the PPARGC1A genotypes with steatosis, activity, and fibrosis in patients with nonalcoholic fatty liver disease

Histological features		Genotypes		Dominant model		Multivariate analysis[1]	
		GG (*n* = 14)	GA + AA (*n* = 45)	*P* value	OR (95%CI)	*P* value	OR (95%CI)
Steatosis (S)	S1	10 (71.43%)	15 (33.33%)				
	S2-3	4 (28.57%)	30 (66.67%)	0.012	5.000 (1.343-18.620)	0.011	6.190 (1.508-25.410)
Activity (A)	A < 2	6 (42.86%)	7 (15.56%)				
	A ≥ 2	8 (57.14%)	38 (84.44%)	0.031	4.071 (1.076-15.402)	0.040	4.506 (1.070-18.978)
Significant fibrosis	F0-1	12 (85.71%)	28 (62.22%)				
	F2-4	2 (14.29%)	17 (37.78%)	0.100	3.643 (0.726-18.292)	0.186	3.449 (0.551-21.598)
Advanced fibrosis	F0-2	13 (92.86%)	35 (77.78%)				
	F3-4	1 (7.14%)	10 (22.22%)	0.206	3.714 (0.432-31.949)	0.426	2.549 (0.255-25.490)
NASH	NAFL	9 (64.28%)	13 (28.89%)				
	NASH	5 (35.71%)	32 (71.11%)	0.017	4.431 (1.245-15.763)	0.035	6.337 (1.135-35.392)

[1]Multivariate analysis adjusted for age, sex, body mass index, and patatin-like phospholipase domain-containing protein 3 rs738409 risk G allele. NAFL: Nonalcoholic fatty liver; NASH: Nonalcoholic steatohepatitis; OR: Odds ratio; CI: Confidence interval.

of adaptive thermogenesis, cellular respiration, and energy metabolism[17]. Recent studies suggested that PGC-1α also regulates lipid metabolism, PGC-1α stimulates the expression of farnesoid X receptor (FXR) target genes in hepatic cells and reduces TG secretion by enhancing FXR activity[18]. In addition, PGC-1α can transactivate the

Table 7 Biochemical and genetic characteristics of patients with nonobese nonalcoholic fatty liver disease

Variables	NAFL (*n* = 6)	NASH (*n* = 12)	*P* value
Sex (M/F)	4/2	10/2	0.569
Age (year)	35.33 ± 18.44	39.67 ± 4.80	0.452
BMI (kg/m^2)	22.83 ± 1.61	23.69 ± 0.90	0.122
ALP (U/L)	123.32 ± 77.62	84.25 ± 23.28	0.426
GGT (U/L)	28.50 (21.18-85.00)	73.25 (54.75-99.25)	0.068
ALT (U/L)	42.00 (21.78-78.50)	71.15 (41.10-115.25)	0.325
TC (mmol/L)	5.11 ± 0.55	4.96 ± 1.02	0.606
TG (mmol/L)	2.00 ± 0.43	1.77 ± 0.95	0.371
HDL-C (mmol/L)	1.10 ± 0.16	1.24 ± 0.44	0.943
LDL-C (mmol/L)	3.16 ± 0.46	2.82 ± 0.80	0.524
FBG (mmol/L)	5.03 ± 0.94	5.24 ± 0.85	0.587
UA (μmol/L)	360.86 ± 167.34	354.17 ± 67.43	0.684
CAP (dB/m)	257.00 ± 57.03	316.73 ± 43.48	0.056
LSM (kPa)	6.95 ± 4.07	6.79 ± 2.06	0.421
PPARGC1A rs8162678			
GG, *n* (%)	4 (66.67)	1 (8.33)	
GA + AA, *n* (%)	2 (33.33)	11 (91.67)	0.021
PNPLA3 rs738409			
CC, *n* (%)	2 (33.33)	1 (8.33)	
CG + GG, *n* (%)	4 (66.67)	11 (91.67)	0.245

The data are expressed as mean ± SD, or as *n* (%) for normally distributed variables and as median (interquartile range) when distribution of the variable was skewed. NAFL: Nonalcoholic fatty liver; NASH: Nonalcoholic steatohepatitis; BMI: Body mass index; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GGT: γ-glutamyl transpeptidase; TC: Total cholesterol; TG: Total triglycerides; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; FBG: Fasting blood glucose; UA: Uric acid; LSM: Liver stiffness measurement; CAP: Controlled attenuation parameter; PNPLA3: Patatin-like phospholipase domain-containing protein 3.

hepatocyte nuclear factor 4α-dependent human hepatic lipase gene promoter to increase the production of hepatic lipase. The PGC-1α is also highly expressed in the liver and coordinates the induction of fatty acid oxidation when fasting. Multiple studies showed that decreased PGC-1α expression might be the mechanism underlying metabolic diseases. Tissue-specific reduction of PGC-1α expression in adipose, liver, and muscle reduced glucose tolerance and insulin sensitivity in mice[19, 20], supporting the hypothesis that decreased PGC-1α expression in insulin-sensitive tissues might contribute to a higher risk of T2DM. PPARGC1A mRNA expression decreased in muscle and adipose tissue of human participants with T2DM[21], and a correlation existed between adipose PGC-1α protein levels and decreased insulin sensitivity[22]. T2DM was also a risk factor for NAFLD. Another reported PPARGC1A polymorphism rs2290602 also decreased the expression of PPARGC1A in patients with NAFLD[6]. Therefore, genetic factors that alter the expression of PGC-1α might participate in the pathogenesis of NAFLD. The present study also indicated that the expression of PPARGC1A was significantly lower in patients who carried the A allele, which supported the hypothesis that decreased PGC-1α expression in steatosis liver tissues might contribute to a higher risk of NAFLD.

Recently, the association between different single nucleotide polymorphisms (SNPs) and metabolic diseases, such as NAFLD, was reported[23]. Therefore, different polymorphisms of PPARGC1A associated with different metabolic diseases were investigated. Iglseder *et al*[24] showed that the -3974T/C (rs2970865) polymorphism of the *PPARGC1A* gene was associated with the severity of carotid atherosclerosis. The variations in rs2290602 in the *PPARGC1A* gene was expected to affect lipid and glucose metabolism and result in the development of NAFLD and NASH[6]. Another

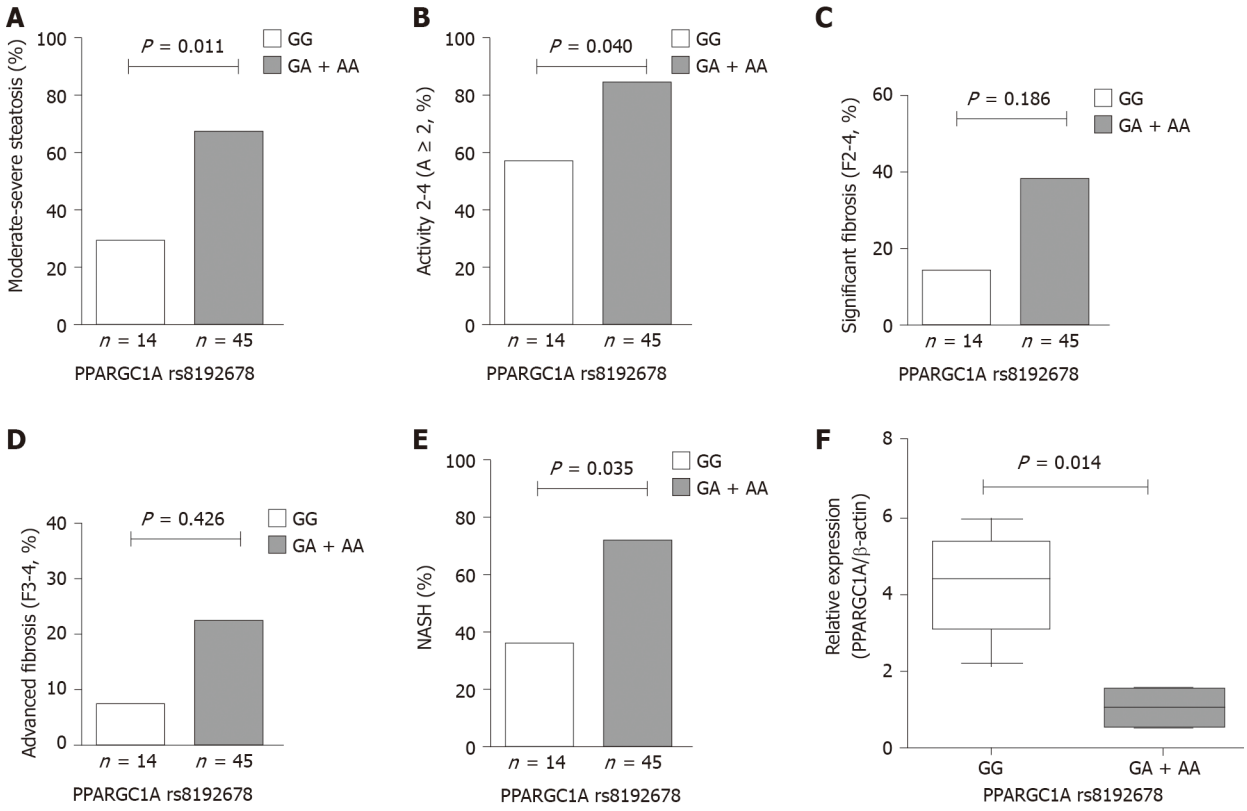


Figure 1 Comparison of various quantitative phenotypes (hepatic histological features and mRNA expression) in patients with nonalcoholic fatty liver disease at the PPARGC1A rs8192678 polymorphism. A-E: Prevalence of moderate-severe steatosis (S2-3), activity 2-4 (A ≥ 2), significant fibrosis (F2-4), advanced fibrosis (F3-4), and nonalcoholic steatohepatitis according to the PPARGC1A rs8192678 polymorphism in 59 patients with nonalcoholic fatty liver disease. The reference genotype for the PPARGC1A rs8192678 polymorphism was GG, and a mode of dominant inheritance was used; F: The intrahepatic mRNA expression of PPARGC1A between the GG group (n = 5) and the GA or AA group (n = 5) at PPARGC1A rs8192678. NASH: Nonalcoholic steatohepatitis.

common PPARGC1A rs8192678 polymorphism might contribute to the risk of coronary artery disease in the Chinese population[9,24], and was also associated with obesity, hypertension, and T2DM. This polymorphism in PPARGC1A was also reported to be related to the susceptibility of NAFLD in different populations and ethnicities. The present study showed that the variation of PPARGC1A rs8192678 GA genotype was significantly higher in patients with NAFLD, but PPARGC1A rs8192678 AA genotype showed no difference between NAFLD and control groups, and the percentage of AA genotype was only 13.56% (8/59) in NAFLD patients, which was consistent with the findings of Lin *et al*[7] in obese children in Taiwan. The explanation of this finding in Lin *et al*[7] concluded two probable reasons. First, the number with PPARGC1A rs8192678 AA genotype was relatively small, because of the sample variation and inadequate statistical power, the role of AA genotype in NAFLD was not presented. Second, the cellular effects of PGC-1α were complicated, and the mechanism by which PGC-1α influenced the pathogenesis of NAFLD was still unclear [7]. However, a comparison between GA + AA and GG genotypes revealed that the participants who carried the PPARGC1A rs8192678 A allele were found to be significantly associated with not only liver biopsy-proven NAFLD but also NASH, independent of age, sex, BMI, and PNPLA3 rs738409 risk G allele in the dominant model. No study analyzed the association between the PPARGC1A rs8192678 A allele and hepatic histological features in NAFLD. Significant associations of PPARGC1A rs8192678 risk A allele with moderate-to-severe steatosis and higher histological activity in patients with NAFLD were found in this study. However, significant fibrosis and advanced fibrosis were not associated with PPARGC1A rs8192678 risk A allele. This suggested that PPARGC1A rs8192678 risk A allele was associated with steatosis and liver damage, contributing to the occurrence of NASH.

Several studies reported that PPARGC1A rs8192678 was significantly associated with obesity; this polymorphism conferred a higher risk of obesity in certain populations[25,26]. The patients who carried PPARGC1A rs8192678 AA genotype benefited more from interventions aimed at weight loss, including caloric restriction,

bariatric surgery, and acarbose treatment[27,28]. The prevalence of lean NAFLD and nonobese NAFLD has increased in eastern and western countries in recent years. The genetic, metabolic, microbiome, metabolomics, and inflammatory factors may also be the causes of NAFLD in lean or nonobese individuals. Genetic factors may also be important in the occurrence and development of nonobese NAFLD. IFNL3 rs12979860 polymorphism has been shown to be associated with hepatic inflammation and fibrosis in patients with nonobese NAFLD[29]. Two SNPs rs12447924 and rs12597002 of the cholesteryl ester transfer protein gene were reported to be associated with a risk of steatosis in nonobese individuals[30]. Wang *et al*[31] found a significantly higher prevalence of advanced fibrosis (F ≥ 3) and LSM values in patients with nonobese NASH compared with obese NASH; also, a higher prevalence of TM6SF2 T allele was observed in patients with nonobese NASH, indicating that patients with nonobese NASH had more severe hepatic histological changes and genetic susceptibility in the Chinese population[31]. The present study found that the PPARGC1A rs8192678 A allele was not significantly closely associated with obese participants, patients with obese NAFLD, and patients with obese NASH. However, the PPARGC1A rs8192678 A allele was relatively more common in patients with nonobese NASH compared with patients with obese NASH. The results also showed that the PPARGC1A rs8192678 GA + AA genotypes were remarkably associated with nonobese NASH, indicating that the PPARGC1A rs8192678 A allele was associated with a high risk of NASH. This, especially, increased the risk of nonobese NASH, and the PPARGC1A rs8192678 polymorphism might be the pathogenesis of nonobese NASH. Despite no significant difference in TC, TG, ALT, GGT, FBG, UA, and CAP values and PNAPL3 rs738409 between the nonobese NAFL and NASH groups, the levels of GGT (P = 0.068) and CAP (P = 0.056) followed an increasing trend in nonobese NASH. This suggested that genetic background might also be a risk factor for nonobese NASH. The influence of metabolic factors, such as lipid and glucose, needs further exploration using a large sample.

The association between PPARGC1A rs8192678 and NAFLD in the Asian population is controversial. Hui *et al*[15] and Zhou *et al*[14] reported no association between the PPARGC1A rs8192678 variant and ultrasound-defined NAFLD in Chinese adults[14,15]. However, Tai *et al*[8] reported that the PPARGC1A variant rs8192678 was associated with liver biopsy-proven NASH in severely obese Taiwanese patients[8]. Saremi *et al*[32] also indicated that the AA genotype and A allele of PPARGC1A increased in Iranian patients with liver biopsy-proven NAFLD[32]. The frequency of the PPARGC1A rs8192678 A allele was 0.453 in Han Chinese, 0.350 in Europeans, and 0.040 in Africans according to the National Center for Biotechnology Information human SNP database, which inferred that the PPARGC1A rs8192678 risk A allele conferred a higher genetic susceptibility to NAFLD in Asians than in Europeans and Africans[7]. Also, the PPARGC1A rs8192678 was associated with NAFLD, especially NASH, in the Chinese Han adult population with liver biopsy-proven NAFLD. This contradictory conclusion might be due to the different criteria used for the definition of NAFLD; ultrasonography or liver biopsy was carried out in different studies. In the present study, the gold diagnostic standard for NAFLD, liver histopathology, was used to avoid the potential image bias in Chinese Han patients with NAFLD.

However, this study also had limitations. First, obtaining liver biopsies was difficult, and hence patients with NAFLD were relatively few, especially those with nonobese NAFLD. Consequently, no statistically significant differences were identified. Second, the risk factors such as hypertension and diabetes, which might influence obese/nonobese NAFLD, were not mentioned in this study. Therefore, a large number of patients with liver biopsy-proven NAFLD should be included in further studies, and well-designed case-control studies should be performed to confirm and support these findings.

CONCLUSION

In conclusion, the present study showed that PPARGC1A rs8192678 risk A allele was associated with liver biopsy-proven NAFLD/NASH, which also had an effect on the severity of hepatic histological features (S2-3 and A ≥ 2), and might also be associated with nonobese NASH in the Chinese Han adult population. These findings also suggested that PPARGC1A polymorphism rs8192678 risk A allele and lower expression of PPARGC1A mRNA might contribute to the etiology of NAFLD. Further studies with large samples are required to confirm these findings.

ARTICLE HIGHLIGHTS

Research background

Nonalcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease and is a significant global burden worldwide. It is also common in obese and nonobese populations. Although the mechanism of NAFLD is unclear, genetic susceptibility plays a vital role in NAFLD. The association between PPARGC1A rs8192678 polymorphism and NAFLD has been reported in several studies, but it is controversial in the Asian population. Whether PPARGC1A rs8192678 is associated with hepatic histological features in NAFLD in the Chinese population is unknown.

Research motivation

The association between PPARGC1A rs8192678 polymorphism and NAFLD requires further investigation, and the association with hepatic histological features and nonobese NAFLD in the Chinese population is unknown.

Research objectives

The aim was to investigate the association between PPARGC1A rs8192678 polymorphism and nonalcoholic steatohepatitis (NASH) and to determine whether PPARGC1A rs8192678 is associated with the hepatic histological features of NASH.

Research methods

Patients with NAFLD and healthy controls were recruited to a cohort representing the Chinese Han population. Patients with NAFLD were proven by liver biopsy. The SAF (Steatosis, activity, and fibrosis) scoring system was used for histopathological evaluation. The polymorphisms of PPARGC1A rs8192678 and patatin-like phospholipase domain-containing protein 3 (PNPLA3) rs738409 were genotyped. The intrahepatic mRNA expression of PPARGC1A was evaluated by real-time polymerase chain reaction.

Research results

In NAFLD patients, 37 patients had NASH, of which 12 were nonobese NASH. The PPARGC1A rs8192678 risk A allele (carrying GA and AA genotypes) increased the risk of NAFLD in the dominant model. The PPARGC1A rs8192678 A allele was also found to be a risk factor for NAFLD after adjusting for age, sex, and PNPLA3 rs738409 polymorphism. In the hepatic histological features of NAFLD patients, moderate-to-severe steatosis (S2-3), and Activity 2-4 (A ≥ 2) were more likely to carry PPARGC1A rs8192678 risk A allele. After adjusting for age, sex, body mass index, and PNPLA3 rs738409 risk G allele, the PPARGC1A rs8192678 risk A allele was also independently associated with S2-3, A ≥ 2, and NASH. The results also showed that this polymorphism was associated with nonobese NASH. In the group of patients who carried A allele (GA or AA genotypes), the intrahepatic expression of PPARGC1A mRNA was significantly lower than that in patients with GG genotype.

Research conclusions

The PPARGC1A rs8192678 A allele is a risk factor for NAFLD, and is associated with the severity of hepatic histological features (S2-3 and A ≥ 2) and NASH in NAFLD patients, independent of PNPLA3 rs738409, and might be associated with nonobese NASH.

Research perspectives

The result that PPARGC1A rs8192678 was associated with liver biopsy-proven NASH and had an additive effect on the severity of hepatic histological features was further confirmed in the Chinese Han adult population. PPARGC1A rs8192678 might contribute to the etiology of NAFLD. PPARGC1A rs8192678 might be a useful tool for diagnosing NASH and predicting the severity of the hepatic histological features of NAFLD.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Does endoscopic intervention prevent subsequent gastrointestinal bleeding in patients with left ventricular assist devices? A retrospective study

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Conflict-of-interest statement:

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Abstract

BACKGROUND

Patients with left ventricular assist devices (LVADs) are at increased risk for recurrent gastrointestinal bleeding (GIB) and repeat endoscopic procedures. We assessed the frequency of endoscopy for GIB in patients with LVADs and the impact of endoscopic intervention on preventing a subsequent GIB.

AIM

To evaluate for an association between endoscopic intervention and subsequent GIB. Secondary aims were to assess the frequency of GIB in our cohort, describe GIB presentations and sources identified, and determine risk factors for recurrent GIB.

METHODS

We conducted a retrospective cohort study of all patients at a large academic institution who underwent LVAD implantation from January 2011 – December 2018 and assessed all hospital encounters for GIB through December 2019. We performed a descriptive analysis of the GIB burden and the outcome of endoscopic procedures performed. We performed multivariate logistic regression to evaluate the association between endoscopic intervention and subsequent GIB.

RESULTS

In the cohort of 295 patients, 97 (32.9%) had at least one GIB hospital encounter.

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Data sharing statement: Statistical code and dataset are available from the corresponding author at sonalipalchaudhuri@gmail.com

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There were 238 hospital encounters, with 55.4% (132/238) within the first year of LVAD implantation. GIB resolved on its own by discharge in 69.8% (164/235) encounters. Recurrent GIB occurred in 55.5% (54/97) of patients, accounting for 59.2% (141/238) of all encounters. Of the 85.7% (204/238) of encounters that included at least one endoscopic evaluation, an endoscopic intervention was performed in 34.8% (71/204). The adjusted odds ratio for subsequent GIB if an endoscopic intervention was performed during a GIB encounter was not significant (odds ratio 1.18, $P = 0.58$).

CONCLUSION

Patients implanted with LVADs whom experience recurrent GIB frequently undergo repeat admissions and endoscopic procedures. In this retrospective cohort study, adherence to endoscopic guidelines for performing endoscopic interventions did not significantly decrease the odds of subsequent GIB, thus suggesting the uniqueness of the LVAD population. A prospective study is needed to identify patients with LVAD at risk of recurrent GIB and determine more effective management strategies.

Key Words: Gastrointestinal bleeding; Left ventricular-assist device; Endoscopic intervention, Inpatient care; Hospital readmissions; Recurrent bleeding

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Core Tip: Patients implanted with left ventricular assist devices (LVADs) whom experience recurrent gastrointestinal bleeding (GIB) frequently undergo repeat admissions and endoscopic procedures. In this retrospective cohort study, a majority of GIB resolved by discharge without intervention and adherence to endoscopic guidelines for performing endoscopic interventions did not significantly decrease the odds of subsequent GIB, thus questioning the role of endoscopy in this population. A prospective study is needed to identify patients with LVAD patients at risk of recurrent GIB and determine more effective management strategies.

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INTRODUCTION

In the last decade, there has been an overall increase in the implantation of continuous-flow left ventricular assist devices (LVADs), a treatment modality for end-stage heart failure as a bridge to transplant, bridge to recovery, or destination therapy (DT). An overall unchanged rate of heart transplantation has resulted in growing cohort of patients with LVADs[1]. Gastrointestinal bleeding (GIB) is one of the most common adverse event in patients with LVADs[2], cited as affecting 21%-36% of patients[2,3], suggested to be due to chronic anticoagulation (AC) and continuous-flow states[4]. The incidence increases with length of time exposed to LVAD.

Prior cohort studies at tertiary care centers, including one at our institution, describe the GIB sources and outcomes of endoscopic evaluations, suggesting that endoscopic interventions are successful in short-term resolution of GI bleeding[3,5]. Meanwhile, multiple studies have shown that up to 30%-60% of patients experience recurrent bleeding (defined as 2 or more episodes) regardless of intervention[3,4], and a large portion of patients require repeat interventions for recurrent bleeding[6]. There are limited data on whether endoscopic intervention reduces recurrent bleeding, bringing into question its utility in managing this chronic issue.

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The primary aim of this study was to evaluate whether endoscopic intervention could prevent a subsequent GIB in patients with LVADs. Secondary aims were to assess the frequency of GIB in our cohort, describe GIB presentations and sources identified, and determine risk factors for recurrent GIB.

MATERIALS AND METHODS

Study population

We performed a retrospective cohort study of all patients ≥ 18 years old who underwent LVAD implantation between January 1, 2011 and December 31, 2018 at our large academic institution. For these 319 patients, we reviewed the electronic medical record (EMR) for demographics, and the date, purpose, and type of LVAD implantation. We excluded patients with temporary devices implanted (CentriMag, Thor BiVAD, Total Artificial Heart), resulting in a total of 295 patients. Data were collected from time of LVAD implantation until death, heart transplant, LVAD explantation, or last contact through the EMR, defined as the number of days followed. This chart review was conducted by two clinical physicians and a medical doctorate-trained research assistant.

For each patient, we reviewed the EMR for hospital encounters from January 1, 2011 through December 31, 2019 to identify instances of GIB on admission or during hospitalization, as indicated in discharge summaries, GI consult notes, and/or endoscopic procedure notes. We included encounters as a GIB if there was overt bleeding reported or documented, or the cardiology team documented suspicion for a GIB based on a drop in hemoglobin or other clinical factors with the lack of other explanation. All encounters with procedures for non-bleeding related indications, like colon cancer screening, or iatrogenic bleeding specifically from prior endoscopic procedures were excluded from analysis. Per standard practice by the cardiology team, patients with concern for GIB are managed in the inpatient setting, so there are no outpatient endoscopic procedures.

For each GIB encounter, we recorded laboratory data, blood transfusion requirements, endoscopic data including video capsule endoscopy findings, and relevant patient medications on admission and discharge (AC, antiplatelet, and octreotide). For encounters when GIB was present on admission, we recorded the length of stay. We classified the GIB presentation as overt *vs* occult, where overt indicated bloody output from the GI tract (*i.e.* hematemesis, hematochezia, melena, coffee-ground emesis), and occult indicated no bloody output visualized but the presence of a hemoglobin drop with no other known etiology.

During a GIB encounter, the primary admitting cardiology team would consult the GI service to determine whether to perform an endoscopic procedure. Medical management regarding acid suppression therapy and octreotide was at the discretion of the cardiology team; standard of care was to continue or initiate acid suppression therapy if concerned for upper GI source and only octreotide if concerned for variceal source. Radiologic studies like computed tomography were performed at the discretion of the cardiology team. All endoscopic procedures were performed with GI endoscopists in the inpatient endoscopy operating room under monitored anesthesia care and the presence of an LVAD coordinator. For patients with elevated international normalized ratio (INR)s, endoscopic procedures aside from video capsule endoscopy (VCE) were performed after an INR normalized to 1.5 or below. The decision to proceed with planned endoscopy based on clinical status of the patient and performance of an endoscopic intervention was at the discretion of the GI endoscopist.

This project was reviewed and determined to qualify as Quality Improvement by the University of Pennsylvania's Institutional Review Board.

Data collection

We reviewed each GIB encounter for endoscopic procedures, including upper endoscopy (EGD) push enteroscopy, single balloon enteroscopy, double balloon enteroscopy, colonoscopy, and VCE. Data were extracted from procedure reports for the presence of a bleeding source and the occurrence of an endoscopic intervention. Endoscopic interventions were defined as epinephrine injection, clip placement, argon plasma coagulation (APC), and bipolar coagulation. For VCE studies, we recorded whether a source was identified and whether an endoscopic procedure occurred afterwards with or without endoscopic intervention.

Study data were collected and managed using Research Electronic Data Capture (REDCap) electronic data capture tools hosted at the University of Pennsylvania[7,8]. REDCap is a secure, web-based software platform designed to support data capture for research studies, providing: (1) An intuitive interface for validated data capture; (2) Audit trails for tracking data manipulation and export procedures; (3) Automated export procedures for seamless data downloads to common statistical packages; and (4) Procedures for data integration and interoperability with external sources.

Power calculation

Based on pilot data suggesting that endoscopic interventions occurred in about a third of encounters, we used a 2:1 allocation ratio. We assumed that endoscopic intervention would decrease the probability of a subsequent GIB from 30% to 50% based on pilot data and what we deemed clinically significant. In order to reject the null hypothesis that endoscopic intervention did not affect subsequent bleeding with a probability (power) of 80% and a type I error probability of 0.05, we needed to study a total of 200 GIB encounters.

Independent (exposure) and dependent (outcome) variables

For the primary aim, the primary outcome variable was a subsequent encounter for GIB. Independent variables included: GIB presentation (overt *vs* occult as defined above), change in AC or antiplatelet therapy, defined as increasing or decreasing doses during the hospitalization or switching agents; days from hospital presentation to endoscopy; source identification during endoscopy or VCE; and endoscopic intervention.

For the secondary aims, the outcome variable was a first GIB. Independent variables included demographics, type of LVAD, purpose of LVAD, and days of exposure to LVAD, defined as the time since LVAD implantation.

Statistical analysis

To evaluate the demographic and clinical differences between those who never had a GIB *vs* those who had at least one, we used a paired *t* test and Wilcoxon rank-sum test for continuous variables that are normally distributed or skewed respectively, and Fisher's exact test for binary and categorical variables. For medians reported, we assessed interquartile range (IQR). We used logistic regression to determine the risk-adjusted impact of endoscopic intervention on subsequent GIB encounters. Risk adjustment variables included age, sex, race, AC status on admission, changes in AC during the admission, source of bleed if applicable, and clinical presentation of GIB. All analyses were performed using STATA version 13.0 (College Station, TX) and reviewed by a biomedical statistician.

RESULTS

Frequency of GIB in patients with LVADs

There was a total of 295 patients who had undergone LVAD implantation during the study period. The devices used were HeartMate2 (57.3%), Heartmate3 (11.2 %), and HeartWare (31.5%). 82.3% were male and median age at time of implant was 58.5 years. Patients were followed for a median of 601 d (IQR 165-1138); there were 120 patients who were followed for less than 1 year due to death, device explantation, or heart transplant.

Of the 295 patients, 97 (32.9%) patients presented for at least one GIB encounter. There was a total of 238 bleed encounters, of which 132 (55.4%) were within the first year of LVAD implantation. Time to index GIB was a median of 132 days (IQR 29-338). 87.4% (208/238) of the GIB encounters were in patients on active AC with either warfarin or a direct oral anticoagulant. Aspirin dose was 81mg daily and 325 mg daily in 25.6% (61/238) and 38.7% (92/238) encounters respectively. The most common presentation of GIB was melena (52.9%), followed by other overt GIB (total 21.4%; hematochezia 11.5%, hematemesis 3.0%, coffee-ground emesis 0.4%, not further characterized 6.5%) and occult bleeding (24.0%). Hemoglobin on presentation was a mean of 7.8 g/dL (IQR 6.3-8.8).

Patient characteristics are described in Table 1 comparing those who had at least one GIB *vs* those without one. Those with GIB were more likely to be older at the time of LVAD implant (age 60.8 *vs* 56.9; $P = 0.01$) and have a higher number of LVAD exposure days (348 *vs* 895, $P < 0.01$). They were also more likely to have the LVAD

Table 1 Comparative characteristics of patients with left ventricular assistant devices with gastrointestinal bleeding *vs* no gastrointestinal bleeding, *n* (%)

Factor		No GIB	At least 1 GIB	*P* value
n		198	97	
Age implant, median (IQR)		56.9 (46.9, 67.0)	60.8 (52.7, 69.8)	0.014
Sex (male)		160 (81.2)	82 (84.5)	0.52
Race	White	113 (57.1)	58 (59.8)	0.067
	Black	54 (27.3)	31 (32.0)	
	Asian	0 (0.0)	1 (1.0)	
	Other	12 (6.1)	5 (5.2)	
	Unknown	19 (9.6)	2 (2.1)	
Type of LVAD	Heartware	66 (33.3)	27 (27.8)	0.22
	HeartMate 2	114 (57.6)	55 (56.7)	
	HeartMate3	18 (9.1)	15 (15.5)	
LVAD purpose	Destination (DT)	113 (57.1)	70 (72.2)	0.030
	bridge to transplant (BTT)	80 (40.4)	25 (25.8%)	
	Bridge to Recovery (BTR)	5 (2.5)	2 (2.1)	
LVAD exposure (d) (IQR)		348 (103, 947)	895 (520, 1433)	< 0.001

The following statistical tests were utilized: Wilcoxon rank-sum test [age, left ventricular-assist device(LVAD)], and Fisher's exact test (sex, race, type of LVAD, LVAD purpose). GIB: gastrointestinal bleeding; IQR: Interquartile range; LVAD: Left ventricular-assist device.

placed as DT (72.2 % *vs* 57.4 %; $P = 0.03$), though in a logistic regression adjusted for the number of days followed, this was no longer significant ($P = 0.14$). Three patients died during active GIB; in two encounters, the patient had a LVAD thrombosis while AC was held, while in the third, the family declined further evaluation of the GIB.

Characteristics of endoscopic procedures and interventions

Of the 238 GIB encounters, 204 (85.7%) included at least one endoscopic evaluation including VCE. After excluding 13 encounters with only VCE evaluation, 191 (80.3%) had an invasive procedure. The median number of endoscopic procedures done per encounter was 2 (range 0 to 8). A source was identified in 130/238 (54.6%) encounters; when identified, the source was in the stomach (41.5%), deep small bowel (jejunum and ileum) (30.8%), colon (13.1%), duodenum (13.1%), and esophagus (1.5%). Of 115 encounters where the first procedure was an EGD, 9 (7.8%) included a push enteroscopy later in the same encounter.

An endoscopic intervention was performed in 34.8% (71/204) of encounters with endoscopic evaluation. The most common lesion intervened upon was angioectasias (Table 2). The second most common lesion intervened upon was non-specific oozing, referring to scenarios where there was no identifiable ulcer, angioectasia, or vessel. Other/uncharacterized category includes cases where the documentation did not express the source in the categorical terms. The most common type of intervention was APC and injection was always in conjunction with another intervention.

Among patients who presented to the hospital for a GIB, 45% of encounters with an endoscopic procedure performed within four days resulted in an endoscopic intervention during the hospitalization, compared to 25% if performed on days 5-7 from presentation, and 0 if performed later. Thus, days to the first endoscopic study impacts whether an endoscopic intervention was performed during the encounter [unadjusted odds ratio (OR) 0.80, confidence interval (CI) 0.67-0.96, $P = 0.018$].

Other predictors of endoscopic intervention included overt GIB compared to occult (OR 2.41, CI 1.14-5.10, $P = 0.022$) and transfusion with packed red blood cells (OR 10.0, CI 1.31-76.47, $P = 0.027$).

In encounters for occult bleeding, a culprit lesion was found in 42.1% of cases (24/57). An endoscopic intervention was performed in 10 of 24 (41.6%) and the attributed source was identified as an angioectasia in eight encounters and as oozing

Table 2 Lesion types and interventions used in endoscopic procedures with interventions, *n* (%).

Category		*n* (%)
Type of intervention	APC	37 (51.4)
	Hemoclip	31 (43.1)
	Injection	23 (31.9)
	Bipolar	16 (22.2)
Culprit lesion	Ulcer	8 (11.1)
	Angioectasia	34 (47.2)
	Dieulafoy	3 (4.2)
	Non-specific oozing	14 (19.4)
	Other/uncharacterized	13 (18.1)

Sum of percentages is greater than 100% as some procedures involved multiple interventions. One encounter had two lesions. APC: Argon plasma coagulation.

without a discrete lesion in the other two encounters. Of 12 encounters where a source was identified in the deep small bowel, six were addressed with endoscopic intervention. Of gastric occult sources, described as erosions, gastropathy, or gastritis, only one (1) underwent intervention.

Excluding the three encounters resulting in death before resolution of GIB, a source was not identified in 46.0% of encounters (108/235), and an intervention was not performed in 69.8% (164/235) encounters, in all of which the GIB resolved on its own by discharge.

Subsequent bleeding: Impact of source identification and endoscopic interventions

Patients who experienced a GIB had a mean of 2.2 encounters for GIB; 55.7% (54/97) of patients with GIB had 2 or more encounters for GIB and 59.2% (141/238) of all encounters were for recurrent GIB (2nd or greater episode). Of all patients with an LVAD, 11.9% (35/295) had 3 or more GIB encounters, resulting in 157 hospitalizations over the span of 7 years (mean 22.4 per year, max 52 in the year 2018).

Table 3 compares the encounter characteristics of GIB encounters with a subsequent GIB *vs* those without, where multiple encounters regarding the same patient are represented individually. There was no statistical difference in age, race, or sex. There was also no statistical difference whether there was a change in AC, if the GIB was overt *vs* occult, or if a source was identified. Endoscopic intervention during an encounter did not significantly impact the odds of a subsequent GIB (adjusted OR 1.18, $P = 0.58$). The median number of days to a subsequent GIB was 78 d (IQR 21-212) and not statistically different between encounters with endoscopic intervention and those without (Table 4). The proportion of encounters with subsequent GIB within 30 d was 29.5% in those with endoscopic intervention and 34.0% for those without, which was also not statistically different ($P = 0.37$). For those with GIB on admission, length of stay was median 12 days (IQR 8-21 d) and not statistically different between encounters with endoscopic intervention and those without ($P = 0.58$).

For subsequent bleeds when a prior source was not identified, a source on the current admission was identified in 20 of 45 (44%). Among the 51 encounters in which a source was identified in both the current GIB and the prior GIB, the source was in the same described area in 36 (70.6%) encounters. Of 22 cases of recurrent bleeding when the prior GIB source was deep small bowel, the current source was also in the deep small bowel in 18; the other 4 encounters sourced the bleed in the duodenum. Of the 28 patients who were found to have a small bowel bleed on at least one encounter, 11 (39.28%) patients had at least one subsequent bleed with a source identified in the small bowel. Otherwise, there was no significant association between the location of bleed identified and the presence of a subsequent bleed.

DISCUSSION

GIB is one of the most common complications in LVAD patients after implantation

Table 3 Comparative characteristics of gastrointestinal bleeding encounters with a subsequent gastrointestinal bleeding *vs* no subsequent gastrointestinal bleeding, *n* (%)

Factor		No subsequent GIB	Had a subsequent GIB	*P* value
n		97	141	
Change in anticoagulation		56 (64.4)	87 (65.4)	0.89
Overt bleed		75 (78.9)	102 (73.4)	0.36
Hemoglobin, median (IQR)		7.8 (6.8, 9.1)	7.5 (6.2, 8.4)	0.043
Source identified		51 (52.6)	79 (56.0)	0.69
Culprit lesion	Ulcer	3 (11)	5 (11)	1.00
	Angioectasia	13 (48)	21 (48)	
	Dieulafoy	1 (4)	2 (5)	
	Non-specific oozing	5 (19)	9 (20)	
	Other	5 (19)	7 (16)	
Culprit lesion location	Esophagus	2 (2.2)	0 (0.0)	0.38
	Stomach	24 (25.8)	30 (22.6)	
	Duodenum	6 (6.5)	11 (8.3)	
	Deep small bowel	12 (12.9)	28 (21.1)	
	Colon	7 (7.5)	10 (7.5)	
	Not identified	42 (45.2)	54 (40.6)	
Endoscopic intervention performed		27 (27.8)	44 (31.2)	0.58
Days to first endoscopic study (mean ± SD)		3.4 ± 7.1	2.9 ± 3.6	0.52

Deep small bowel refers to jejunum and ileum. The following statistical tests were utilized: paired *t* test (days to first endoscopic study), Wilcoxon rank-sum test (age, hemoglobin), and Fisher's exact test (change in anticoagulation, overt bleed, culprit lesion, culprit lesion location, endoscopic intervention performed). GIB: Gastrointestinal bleeding; IQR: Interquartile range; SD: Standard deviation.

Table 4 Outcomes for gastrointestinal bleeding encounters with endoscopic intervention *vs* none gastrointestinal bleeding

Factor	Endoscopic intervention	No endoscopic intervention	*P* value
Median number of days to subsequent GIB (IQR)	113 (15-302)	72 (24-178)	0.51
	n = 44	*n* = 97	
Proportion with subsequent GIB within 30 days	29.5%	34.0%	0.37
	n = 44	*n* = 97	
Median length of stay in days for those with GIB on admission (IQR)	12 (10-23)	12 (8-21)	0.58
	n = 31	*n* = 86	

The following statistical tests were utilized: Wilcoxon rank-sum test [days to subsequent gastrointestinal bleeding (GIB), length of stay], and Fisher's exact test (proportion with subsequent GIB within 30 d). GIB: Gastrointestinal bleeding; IQR: Interquartile range.

and has become a frequent cause of hospitalization for this population. Patients may have multiple bleeding episodes and nearly 10% of LVAD patients will have 3 or more encounters for GIB. This is the first study to our knowledge that is powered to evaluate whether endoscopic intervention reduces the risk for subsequent GIB. Our results confirm that a high proportion of GIB in our LVAD population clinically stops without endoscopic therapy and that endoscopic intervention does not prevent subsequent bleeding.

Our cohort size is within the wide range of sizes of studied cohorts at other tertiary care centers in terms of the numbers of patients with LVAD implantations and of the encounters for GIB bleeding[3,9,10]. Median time to bleed varies in the prior literature from 55 d to 197 d[3,9,10], where our composite median time of 129 d may reflect a higher proportion of late and recurrent GIB and fewer early GIBs. We found similar factors that correlated with GIB and findings: age correlated with having a GIB[9,11], while overt presentation and need for transfusion support correlated with performing endoscopic interventions[11]. The highest diagnostic yield was confirmed for upper procedures including EGD and push enteroscopy[9,11]. We also found a high burden of GIB caused by angioectasias[5,9]. While Dakik *et al*[6] and Axelrad *et al*[9] found that hemostatic therapy during an index examination was a statistically significant risk factor for a subsequent GIB, this was not the case in our cohort that had more GIB encounters and was followed for a longer period of time. Several factors may have had an impact on this disparity, including cohort size, efficacy of operator specific treatment techniques, selection of significant lesions, nuances in timing, or subtleties in patient demographics.

There are several areas that still warrant further investigation. First, it is unclear if the suboptimal initial diagnostic yield is attributable to delay in endoscopy, impediments imposed by anticipation of INR normalization or completion of bowel preparation, or intermittent visibility and bleeding of lesions such as angioectasias. These variabilities may explain why subsequent GIB encounters are able to isolate a source in some encounters. This low rate of identifying a source is consistent with other studies [9]. While source identification or endoscopic intervention on a visualized lesion did not result in reducing subsequent GIB or readmission rates, other benefits to endoscopy such as shorter length of stay may exist, although we did not find this in our cohort. Addressing these issues may be important in improving the success of endoscopic intervention.

Our findings indicate that minimizing endoscopic utilization may be beneficial for patients and healthcare utilization. There are a few proposed solutions to reduce the burden of low-yield procedures and reduce delay to source identification. Axelrad *et al* [12] proposed an endoscopic algorithm that consisted of push enteroscopy, instead of EGD, or colonoscopy for overt signs of bleeding, along with conservative management without endoscopic evaluation for occult bleeding, and found in a retrospective analysis that this method would improve resource utilization and limit lower-yield procedures. While VCE is often a second line study for persistent bleeding after negative upper and lower endoscopic evaluation[3], Marya *et al*[13] determined that early VCE compared to standard approaches to endoscopy in patients presenting with non-hematemesis GIB increased source localization, with no difference in direct costs of hospitalization. We propose that the VCE be performed urgently in the acute setting while awaiting normalization of the INR prior to possible endoscopy, especially when an endoscopic evaluation has been performed on a prior encounter. This proposal may obviate unnecessary endoscopies should the VCE exclude a targetable lesion, or help identify the most appropriate and high-yield procedure (EGD, push enteroscopy, or balloon enteroscopy) for treatment of an accessible lesion or indicative presence of blood. The yield and cost effectiveness of this strategy deserves further study.

Alternatively, intervention with endoscopic techniques may simply be an inappropriate long-term approach to treatment of certain common hemorrhagic lesions such as angioectasias, which likely represent a systemic process rather than a cluster of focal endoscopic lesions. Designing a randomized trial which withheld endoscopic intervention from some patients with GIB may be impractical given the unclear criteria by which to exclude higher risk patients. However, the creation of a prognostic risk score for patients with LVAD-related GIB could help triage low and high risk patients to different care pathways.

More importantly, recognizing that endoscopy may be only a temporizing measure, there is an urgent need for utilization of medical management protocols to prevent recurrent bleeding. Angioectasia recidivism after endoscopic therapy is common, and endoscopy is rarely a long term solution. Early data suggest that blockade of the angiotensin II receptor activation with angiotensin-converting enzyme inhibitors (ACE inhibitors) or angiotensin receptor blockers may reduce GIB episodes by reducing angioectasia formation[14]. Yin *et al*[10] built a model to predict who may have recurrent bleeding based on age and comorbidities while Welden *et al*[15] found better INR control and early endoscopy within 48 h of admission as clinical predictors of reduced recurrent GIB[15]. AC regimens need better study and standardization to best balance the risks of bleeding and thrombosis[16].

Collaboration amongst gastroenterologists and cardiologists is paramount for achieving optimal patient care, including the management of anti-platelet and AC therapies, LVAD pump speed adjustments, and endoscopic guidance. Alternative pharmaceutical strategies for reducing GIB in this population include the use of octreotide, thalidomide in restricted populations, and desmopressin (which increases the risk for thrombosis); ultimately, cardiac transplantation significantly reduces future GIB, when pulsatile flow and other parameters have been restored[17].

There are several limitations to our study. Our cohort was adequately powered to evaluate for a significant relationship between endoscopic intervention and subsequent GIB, but a larger patient cohort may be able to find statistical significance for a subpopulation, such as those with overt *vs* occult bleeding or with specific sources of bleeding. The retrospective and observational nature of our study limited the ability to show an impact of other parameters like change in AC or change in acid suppression therapy upon admission. Data abstraction was limited for some earlier encounters, including comorbidities and general health condition, due to an interim transition in EMR. There are other factors that contribute to the decisions for GIB management by the individual cardiologists and gastroenterologists that may not have been captured on chart review. We were able to evaluate for GIB encounters within our hospital system and may have missed other GIB admissions, though we think this to be an infrequent scenario as these patients are closely followed by our cardiology colleagues.

CONCLUSION

Patients with LVAD implantation are at high risk for recurrent GIBs. A majority of episodes of GIB resolve without endoscopic intervention. While endoscopic intervention for GIB is the established practice for the general population, our data do not support this position for patients with LVAD. The percentage of patients who had recurrent bleeding after endoscopic intervention was not statistically different than in those who had no intervention (P = 0.92). The rational and focused use of endoscopy, as part of an algorithm for GIB tailored specifically to patients with LVAD, is paramount for providing optimal patient care, limiting risks, and using resources most effectively. We advocate for more prospective evidence which will help support the creation of an evidence-based protocol to manage recurrent GIB and prevent future occurrences.

ARTICLE HIGHLIGHTS

Research background

Patients with left ventricular assist devices (LVADs) are at increased risk for recurrent gastrointestinal bleeding (GIB) and repeat endoscopic procedures.

Research motivation

There are limited data on whether endoscopic intervention reduces recurrent bleeding, bringing into question its utility in managing this chronic issue.

Research objectives

Our primary aim was to evaluate for an association between endoscopic intervention and subsequent GIB. Secondary aims were to assess the frequency of GIB in our cohort, describe GIB presentations and sources identified, and determine risk factors for recurrent GIB.

Research methods

We conducted a retrospective cohort study of all patients at a large academic institution who underwent LVAD implantation from January 2011 – December 2018 and assessed all hospital encounters for GIB through December 2019. We performed a descriptive analysis of the GIB burden and the outcome of endoscopic procedures performed. We performed multivariate logistic regression to evaluate the association between endoscopic intervention and subsequent GIB.

Research results

In the cohort of 295 patients, 97 (32.9%) had at least one GIB hospital encounter and recurrent GIB occurred in 55.5% (54/97) of patients. There were 238 hospital encounters, and GIB resolved on its own by discharge in 69.8% encounters. Of the 85.7% (204/238) of encounters that included at least one endoscopic evaluation, an endoscopic intervention was performed in 34.8% (71/204). The adjusted odds ratio for subsequent GIB if an endoscopic intervention was performed during a GIB encounter was not significant (odds ratio 1.18, $P = 0.58$).

Research conclusions

In this retrospective cohort study, adherence to endoscopic guidelines for performing endoscopic interventions did not significantly decrease the odds of subsequent GIB, thus suggesting the uniqueness of the LVAD population.

Research perspectives

A prospective study is needed to identify patients with LVAD at risk of recurrent GIB and determine more effective management strategies.

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ORIGINAL ARTICLE

Retrospective Study

Diverse expression patterns of mucin 2 in colorectal cancer indicates its mechanism related to the intestinal mucosal barrier

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Author contributions: Liu J and Zheng YF contributed to the conception and design of the study; Gan GL and Wu HT performed most of the experiments, analyzed the data, and drafted and revised the manuscript carefully; Chen WJ, Li CL, Ye QQ, and Zheng YF performed some of the experiments and interpreted the data; Wu HT, Zheng YF, and Liu J provided essential reagents; Liu J assisted with the experimental design and data analysis, and revised the original manuscript critically; all authors contributed to manuscript revision, and read and approved the submitted version.

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Abstract

BACKGROUND

Abnormal expression patterns of mucin 2 (MUC2) have been reported in a variety of malignant tumors and precancerous lesions. Reduced MUC2 expression in the intestinal mucosa, caused by various pathogenic factors, is related to mechanical dysfunction of the intestinal mucosa barrier and increased intestinal mucosal permeability. However, the relationship between MUC2 and the intestinal mucosal barrier in patients with colorectal cancer (CRC) is not clear.

AIM

To explore the relationship between MUC2 and intestinal mucosal barrier by characterizing the multiple expression patterns of MUC2 in CRC.

METHODS

Immunohistochemical staining was performed on intestinal tissue specimens from 100 CRC patients, including both cancer tissues and adjacent normal tissues. Enzyme-linked immunosorbent assays were performed on preoperative sera from 66 CRC patients and 20 normal sera to detect the serum levels of MUC2, diamine oxide (DAO), and D-lactate (D-LAC). The relationship between MUC2 expression and clinical parameters was calculated by the χ^2 test or Fisher's exact test. Prognostic value of MUC2 was evaluated by Kaplan-Meier curve and log-rank

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Institutional review board statement: The current study was reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Shantou University Medical College.

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: No conflict of interest is claimed by any author.

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Grade D (Fair): 0

tests.

RESULTS

Immunohistochemical staining of 100 CRC tissues showed that the expression of MUC2 in cancer tissues was lower than that in normal tissues (54% *vs* 79%, $P < 0.05$), and it was correlated with tumor-node-metastasis (TNM) stage and lymph node metastasis in CRC patients ($P < 0.05$). However, the serum level of MUC2 in CRC patients was higher than that in normal controls, and was positively associated with serum levels of human DAO ($\chi^2 = 3.957$, $P < 0.05$) and D-LAC ($\chi^2 = 7.236$, $P < 0.05$), which are the biomarkers of the functional status of the intestinal mucosal barrier. And the serum level of MUC2 was correlated with TNM stage, tumor type, and distant metastasis in CRC patients ($P < 0.05$). Kaplan-Meier curves showed that decreased MUC2 expression in CRC tissues predicted a poor survival.

CONCLUSION

MUC2 in tissues may play a protective role by participating in the intestinal mucosal barrier and can be used as an indicator to evaluate the prognosis of CRC patients.

Key Words: Colorectal cancer; Mucin 2; Mucin; Expression; Intestinal mucosal barrier; Prognosis

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Core Tip: This study found that mucin 2 (MUC2) in intestinal tissues may play a protective role on the intestine and can be used as one of the indicators to evaluate the prognosis of patients with colorectal cancer (CRC). When the intestinal mucosal barrier function of patients with CRC is impaired, the serum level of MUC2 can reflect the severity of the damage. Therefore, in CRC patients with impaired intestinal mucosal barrier function, the serum level of MUC2 could reflect the severity of the damage, providing a potential mechanism for the development of therapeutic strategies for CRC patients.

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INTRODUCTION

The incidence of colorectal cancer (CRC) ranks third in the world among the common malignant tumors, and the mortality ranks second[1]. Due to large changes in lifestyle and dietary habits, the incidence and mortality of CRC will continue to rise. In China, there are 300000 new CRC cases each year, with an annual average increase of 4.2%[2]. There are obvious gender and regional differences in the incidence and mortality rate of CRC cases, with the overall distribution being more in males than in females, and more in urban areas than in rural areas[3]. Therefore, CRC is one of the major diseases that seriously threatens human life and health, and the situation is quite serious.

The etiological mechanism of CRC tumorigenesis and development is extremely complex, involving a variety of genetic and environmental factors[4]. Among them, the impairment of intestinal barrier structure and function leads to a series of pathophysiological changes in the intestinal mucosa, which eventually evolves into tumor malignancy. Mucins (MUCs) are the main components of the mucus layer, which provides the first-line defense against infection and participates in the process of intercellular adhesion, intercellular communication, and immune regulation[5].

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Among 27 reported MUC proteins, MUC2 is a secretory mucin involved in the formation of mucus, and is mainly secreted by goblet cells[6]. Reduced MUC2 expression in the intestinal mucosa, caused by various pathogenic microorganisms and/or toxic substances, induces apoptosis of intestinal mucosal epithelial cells and destroys the mechanical barrier of the intestinal mucosa, ultimately leading to increased intestinal mucosal permeability[7]. Interestingly, an abnormal expression pattern of MUC2 has been reported in a variety of malignant tumors and precancerous lesions, suggesting an important role for MUC2 in the occurrence and development of CRC[8, 9]. However, the expression of MUC2 is tissue- and cell-specific[10]. Kasprzak *et al*[8] reported a distinct expression pattern of MUC2 in mucinous *vs* non-mucinous colorectal adenocarcinoma[8], suggesting that diverse and specific mechanisms are involved in different types of CRC.

To verify the diverse expression patterns of MUC2 in CRC patients, the current study enrolled CRC patients and investigated MUC2 levels in both malignant tissue and serum to provide an underlying mechanism of MUC2 related to intestinal barrier function, and lay a foundation for further revealing the molecular mechanism of MUC2 involvement in the malignant biological behavior of CRC.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Ethics Committee of the First Affiliated Hospital of Shantou University Medical College. All patients or their guardians signed written informed consent before the study. All experiments were conducted following the guidelines of the Ethics Review Committee.

Patient information

Cancer tissues and adjacent normal intestinal mucosal tissues (> 5 cm away from the tumor) were collected from 100 CRC patients who underwent radical resection at the Department of Gastrointestinal Surgery of the First Affiliated Hospital of Shantou University Medical College from January 2015 to December 2016. The inclusion criteria included: (1) Age from 18 to 90; (2) No medical history of blood, cardiovascular, or immune disease, or inflammatory bowel disease; (3) Pathological diagnosis of CRC; (4) Tumor-node-metastasis (TNM) stages I to III; and (5) Patients who underwent radical surgery. The exclusion criteria included: (1) Preoperative history of neoadjuvant chemotherapy or radiotherapy; (2) Binary or multivariate cancer; (3) Preoperative complications, such as malignant intestinal obstruction, perforation, or bleeding; (4) No standardized chemotherapy after the radical surgery; and (5) Incomplete clinicopathological data.

Peripheral blood was collected before surgery from 66 CRC patients who were diagnosed with CRC at the Department of Gastrointestinal Surgery of the First Affiliated Hospital of Medical College of Shantou University from January 2018 to December 2019. The inclusion criteria were almost the same as above, except that patients at stage IV were also recruited. The exclusion criteria were the same. For comparison, 20 normal subjects in the same period were recruited and their sera were collected in the Health Management Center of the First Affiliated Hospital of Shantou University Medical College.

Histology and immunohistochemistry

Formalin-fixed and paraffin-embedded CRC tissues were cut into 4-μm sections and stained with hematoxylin and eosin. Histopathological differentiation was made by two pathologists based on the World Health Organization criteria. TNM pathological staging was determined according to the staging manual of the American Joint Committee on Cancer[11].

Immunohistochemistry (IHC) was conducted as described before[12]. CRC and adjacent normal tissues were dewaxed in xylene, hydrated in a series of graded alcohols, and placed in a citric acid buffer for epitope retrieval. After immersion in 3% H_2O_2 solution to block endogenous peroxidase, the slides were incubated with anti-MUC2 monoclonal antibody (ab118964, Abcam, United Kingdom) at 4 ℃ overnight. Negative controls were treated with PBS instead of primary antibody. Haematoxylin was used for counterstaining.

Sections were visualized under a bright-field microscope (CKX41, Olympus, Japan) and evaluated independently by two investigators with no prior knowledge of the patient information. For tissue expression of MUC2, staining intensity was recorded as

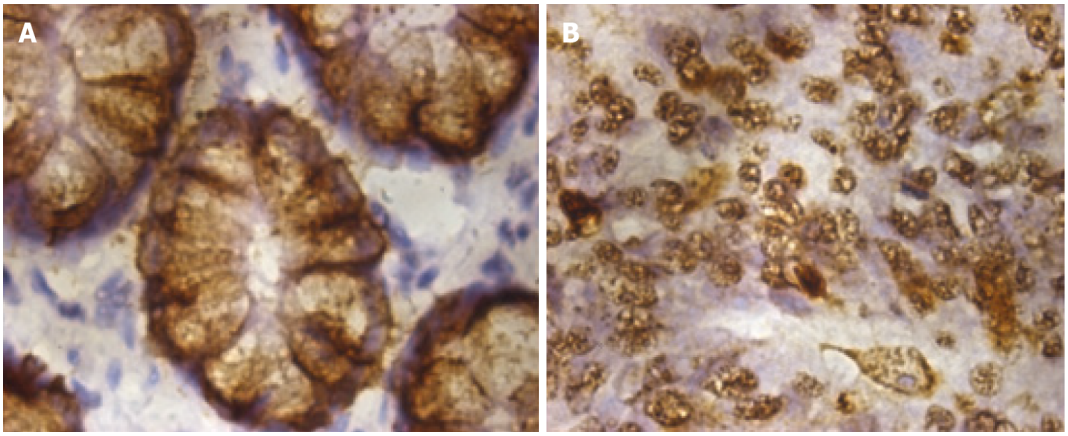


Figure 1 Mucin 2 is mainly located in the cytoplasm. A: Normal tissue; B: Colorectal cancer tissue. Magnification: × 1000.

0, 1, 2 and 3 for colorless, light yellow, brown yellow, and dark brown, respectively, and the percentage of positive cells < 5%, 6%-30%, 31%-60%, and 61%-100% was scored as 0, 1, 2, and 3 points, respectively. To evaluate the expression level of MUC2, the scores of staining intensity and the percentage of positive cells were added and defined as low expression (0-3) and high expression (4-6).

Enzyme-linked immunosorbent assay

The collected peripheral blood samples were centrifuged at 2000-3000 rpm for 15 min, and the supernatant serum was collected carefully and stored in -80 ℃. Human MUC2, human diamine oxide (DAO), and human D-lactate (D-LAC) enzyme-linked immunosorbent assay detection kits were purchased from Nanjing Senberga Biotechnology Co., Ltd., China. Experiments were carried out as previously described[13]. The standard curve was determined by the standard concentration and their corresponding absorbance (OD value). The actual concentrations of MUC2, DAO, and D-LAC were calculated based on the standard curves.

Follow-up and statistical analysis

Overall survival (OS) time was calculated in months from the date of diagnosis to the date of death of the patient or the last follow-up visit. Disease-free survival (DFS) time was determined by the date of relapse.

SPSS 21.0 statistical software was used to analyze the data. Enumerated data are expressed by the number of cases (N), and the measurement data that conformed to a normal distribution are expressed as the mean ± SD. The relationship between MUC2 and the patient's clinicopathological data was tested by χ^2 and Fisher's exact probability tests. The relationship between serum MUC2, DAO, and D-LAC was analyzed by the χ^2 test. The Kaplan-Meier survival curve and log-rank test were used to evaluate the association of MUC2 expression with prognosis. The difference between two groups was statistically significant at $P < 0.05$.

RESULTS

Expression of MUC2 is decreased in cancer tissues compared with normal tissues in CRC patients

To detect the location of MUC2 in intestinal tissues, IHC was performed and revealed that MUC2-positive staining was mainly located in the cytoplasm, both in normal tissues and CRC tissues (Figure 1). In normal tissues, the cytoplasm was diffusely and homogeneously positive, and the nucleus was not stained, while in cancer tissues, cells lost their normal morphology, adenoid structures were destroyed or had even disappeared, and heterotypic cancer cells could be detected.

Representative images of MUC2 staining in adjacent and cancer tissues are shown in Figure 2. Interestingly, the percentage of tissues with high MUC2 expression was significantly decreased from 79% in adjacent normal tissues to 54% in CRC tissues (Figure 3). The difference in MUC2 expression between normal and cancer tissues was statistically significant ($P < 0.01$; Table 1)

Table 1 Comparison of mucin 2 expression in normal and cancer tissues of patients with colorectal cancer (cases)

Group	MUC2 expression		χ^2	*P* value
	Low	High		
Normal tissues	21	79	14.028	< 0.01[1]
Cancer tissues	46	54		

[1]Indicates that the difference was statistically significant, which confirmed that the expression difference of mucin 2 (MUC2) in normal tissues and cancer tissues was statistically significant, and the high expression rate of MUC2 in cancer tissues was lower than that in normal tissues. MUC2: Mucin 2.

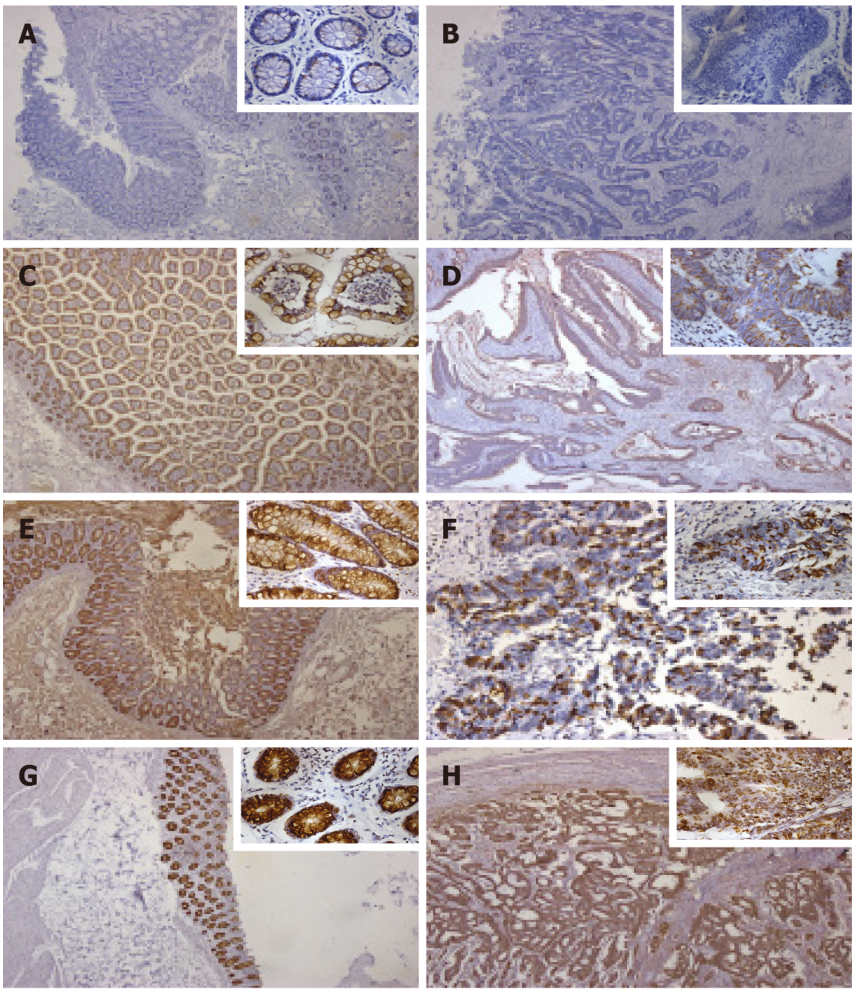


Figure 2 Representative pictures of immunohistochemical staining for mucin 2 in normal and cancer tissues of patients with colorectal cancer. A and B: Negative expression; C and D: Weak expression; E and F: Moderate expression; G and H: Strong expression. A, C, E, and G: Normal tissue; B, D, F, and H: Cancer tissue. Magnification: × 100 and × 400 (located in the upper right corner of each image).

Tissue MUC2 level is negatively associated with TNM stage and lymph node status in patients with CRC

Clinicopathological analysis showed that tissue MUC2 expression (low *vs* high) was not significantly associated with the age at diagnosis, gender, tumor location, tumor size, depth of invasion, degree of differentiation, or tumor type (Table 2). In comparison with tumors at stage III, tumors at stages I and II showed significantly more tissue MUC2 expression (χ^2 = 13.963, P < 0.05). Importantly, low expression of tissue MUC2 was more frequently observed in patients with lymph node metastasis (χ^2 = 12.538, P < 0.05) (Table 2).

Table 2 Relationship between expression of mucin 2 and clinicopathological parameters in patients with colorectal cancer, *n* (%)

Clinicopathological parameter	*n* (%)	MUC2 expression		χ^2	*P* value
		Low, *n* (%)	High, *n* (%)		
Age, yr					
≤ 60	48	22 (45.8)	26 (54.2)	0.001	0.974
> 60	52	24 (46.2)	28 (53.8)		
Gender					
Male	52	23 (44.2)	29 (55.8)	0.137	0.712
Female	48	23 (47.9)	25 (52.1)		
Tumor location					
Rectum and anus	48	21 (43.8)	27 (56.3)	0.188	0.664
Colon	52	25 (48.1)	27 (51.9)		
TNM stage					
I-II	57	17 (29.8)	40 (70.2)	13.963	< 0.01
III	43	29 (67.4)	14 (32.6)		
Maximum tumor diameter					
< 5 cm	49	23 (46.9)	26 (53.1)	0.034	0.854
≥ 5 cm	51	23 (45.1)	28 (54.9)		
Depth of invasion					
Non-immersed serosa	22	8 (36.4)	14 (63.6)	1.054	0.304
Immersed serosa	78	38 (48.7)	40 (51.3)		
Degree of differentiation					
High-moderate	93	42 (45.2)	51 (54.8)	F[1]	0.700
Low	7	4 (57.1)	3 (42.9)		
Tumor type					
Mucinous adenocarcinoma	13	5 (38.5)	8 (61.5)	0.342	0.559
Non-mucinous adenocarcinoma	87	41 (47.1)	46 (52.9)		
Lymph node metastasis					
No	56	17 (30.4)	39 (69.6)	12.538	< 0.01
Yes	44	29 (65.9)	15 (34.1)		

[1]Fisher exact test was used when the expected frequency was less than 1. Due to the small number of patients with stage I, in order to reduce bias, we combined the stage I with stage II patients for analysis. Similarly, there was only one patient with highly differentiated colorectal cancer, so the patient was combined with patients with moderately differentiated disease. It can be seen from the above table that the expression of mucin 2 is correlated with tumor-node-metastasis stage and lymph node metastasis in colorectal cancer patients. MUC2: Mucin 2; TNM: Tumor-node-metastasis.

Serum levels of MUC2 are elevated in CRC patients, and positively associated with the levels of DAO and D-LAC in serum

Serum DAO and D-LAC are indicators of intestinal mucosal barrier permeability and integrity[14,15]. As expected, in patients with CRC, the serum levels of DAO and D-LAC were significantly increased compared with those in normal controls (Figure 4A and B), indicating impaired intestinal mucosal barrier function and increased intestinal permeability in CRC patients (Table 3). Interestingly, serum levels of MUC2 were also higher than those in normal controls (Figure 4C) and closely related to the serum levels of DAO and D-LAC (Table 4).

Table 3 Comparison of serum levels of diamine oxide, D-lactate, and mucin 2 between colorectal cancer patients and normal control (mean ± SD)

Group	*n* (%)	DAO (pg/mL)	D-LAC (μg/L)	MUC2 (ng/L)
Normal control	20	158.21 ± 15.98	973.69 ± 128.08	305.98 ± 31.50
CRC	66	185.40 ± 25.49	1216.93 ± 204.20	364.58 ± 48.30
P value		< 0.01	< 0.01	< 0.01

There were 66 cases of colorectal cancer (CRC) and 20 cases of normal controls. Serum levels of mucin 2, diamine oxide, D-lactate in CRC patients were higher than those in the normal control group. MUC2: Mucin 2; DAO: Diamine oxide; D-LAC: D-lactate; CRC: Colorectal cancer.

Table 4 Relationship between serum levels of mucin 2, diamine oxide, and D-lactate in patients with colorectal cancer (cases)

MUC2	DAO		D-LAC	
	Low	High	Low	High
Low	15	8	16	7
High	17	26	15	28
χ^2	3.957		7.236	
P value	0.047		0.007	

The measured mucin 2 (MUC2) level of P95 in the normal population was taken as the normal reference range, and those beyond P95 were regarded as increased, otherwise as decreased. Based on this, colorectal cancer patients were divided into groups with high and low levels of MUC2. Similarly, the number of cases in diamine oxide (DAO) and D-lactate (D-LAC) groups with high and low levels could be obtained. Serum MUC2 levels were positively correlated with DAO and D-LAC levels. Low: Low serum level; High: High serum level; MUC2: Mucin 2; DAO: Diamine oxide; D-LAC: D-lactate.

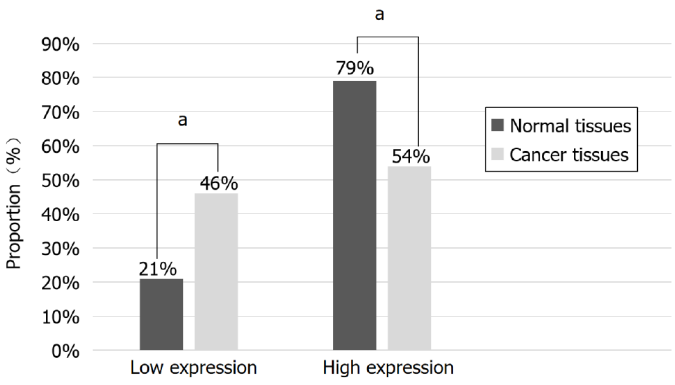


Figure 3 Histogram of mucin 2 expression in cancer and normal tissues of patients with colorectal cancer. According to the comprehensive score of staining intensity and positive cell percentage, 0-3 was classified as low expression and 4-6 as high expression. $^aP < 0.05$.

Serum MUC2 is positively associated with TNM stage and distant metastasis in patients with CRC

As shown in Table 5, the higher the TNM stage in CRC patients, the higher the serum MUC2 level ($P = 0.033$). Importantly, the percentage of patients with high serum levels of MUC2 was dramatically increased in CRC patients with distant metastasis compared with those without (100.0% *vs* 59.6%, $P = 0.022$). The tumor type was also found to be related to the expression of serum MUC2 in CRC patients. CRC patients with mucinous adenocarcinoma had higher serum MUC2 levels than non-mucinous CRC patients ($P < 0.01$). However, serum MUC2 level was not associated with age at diagnosis, gender, tumor size, tumor location, depth of invasion, degree of differentiation, or lymph node metastasis (Table 5).

Low expression of MUC2 in cancer tissues predicts a poor survival in CRC patients

Kaplan-Meier curve analyses with log-rank test revealed that tissue MUC2 expression was significantly associated with DFS ($P = 0.032$) (Figure 5A) and OS ($P = 0.037$)

Table 5 Relationship between serum levels of mucin 2 and clinicopathological parameters in patients with colorectal cancer, *n* (%)

Clinicopathological parameter	*n* (%)	Serum MUC2 expression		χ^2	*P* value
		Low, *n* (%)	High, *n* (%)		
Age, yr					
≤ 60	30	11 (36.7)	19 (63.3)	0.080	0.777
> 60	36	12 (33.3)	24 (66.7)		
Gender					
Male	38	12 (31.6)	26 (68.4)	0.422	0.516
Female	28	11 (39.3)	17 (60.7)		
Tumor location					
Rectum and anus	24	10 (47.1)	14 (58.3)	0.772	0.380
Colon	42	13 (31.0)	29 (65.2)		
TNM stage					
I-II	36	16 (44.4)	20 (55.6)	6.687	0.033
III	21	7 (33.3)	14 (66.7)		
IV	9	0 (0.0)	9 (100.0)		
Maximum tumor diameter					
< 5 cm	33	12 (36.4)	21 (63.6)	0.067	0.796
≥ 5 cm	33	11 (33.3)	22 (66.7)		
Depth of invasion					
Non-immersed serosa	12	5 (41.7)	7 (58.3)	0.300	0.584
Immersed serosa	54	18 (33.3)	36 (66.7)		
Degree of differentiation					
High-moderate	60	21 (38.3)	39 (61.7)	F[1]	1.000
Low	6	2 (33.3)	4 (66.7)		
Tumor type					
Mucinous adenocarcinoma	14	8 (57.1)	6 (42.9)	21.241	< 0.01
Non-mucinous adenocarcinoma	52	15 (32.7)	37 (67.3)		
Lymph node metastasis					
No	36	16 (44.4)	20 (55.6)	3.212	0.073
Yes	30	7 (23.3)	23 (76.7)		
Distant metastasis					
No	57	23 (40.4)	34 (59.6)	F[1]	0.022
Yes	9	0 (0.0)	9 (100.0)		

[1]Fisher exact test was used when the expected frequency was less than 1. Due to the small number of patients with stage I, in order to reduce bias, we combined the stage I with stage II patients for analysis. Similarly, there was only one patients with highly differentiated colorectal cancer, so this patient was combined with the patients with moderately differentiated disease. Serum levels of mucin 2 was correlated with tumor-node-metastasis stage, tumor type, and distant metastasis in colorectal cancer patients. MUC2: Mucin 2; TNM: Tumor-node-metastasis.

(Figure 5B) in all CRC patients. Decreased tissue MUC2 level predicted a poor prognosis of CRC patients. During the 5-year follow-up period, the recurrence was 40.0% in patients with low expression of MUC2 and 18.5% in patients with high expression of MUC2 ($\chi^2 = 5.485$, $P < 0.05$) (Figure 5C).

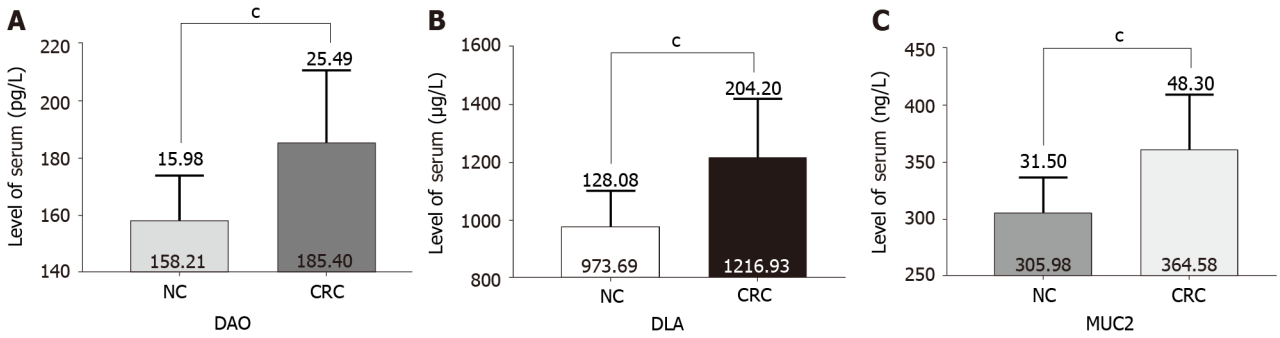


Figure 4 Comparison between the levels of serum diamine oxide, D-lactate, and mucin 2 in colorectal cancer patients and normal controls. A: The level of serum diamine oxidase in colorectal cancer (CRC) patients and normal controls (NC); B: The level of serum D-lactate in CRC patients and NC; C: The level of serum mucin 2 in CRC patients and NC. The numbers at the bottom of the bar chart represent the mean, and the numbers above the error line represent the standard deviation. °$P < 0.001$. CRC: Colorectal cancer; NC: Normal controls; DAO: Diamine oxidase; D-LAC: D-lactate; MUC2: Mucin 2.

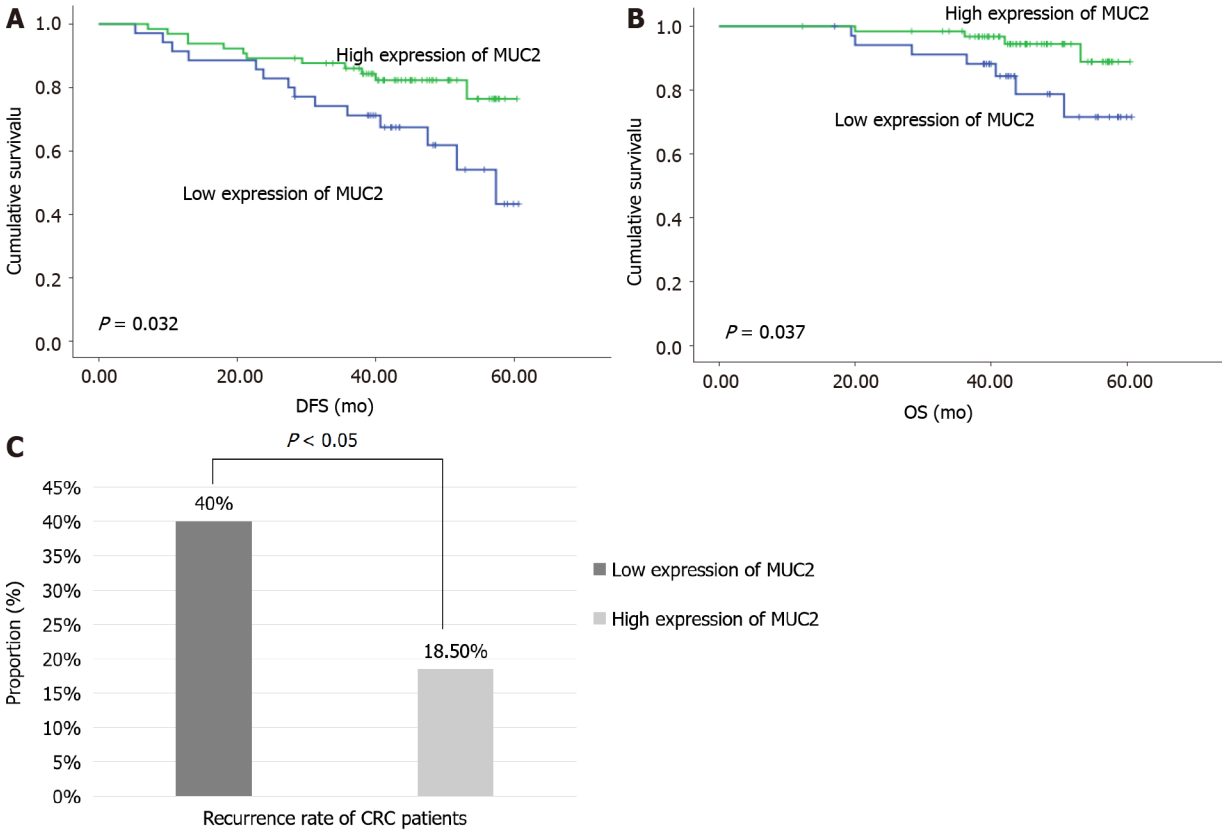


Figure 5 Prognostic value of tissue mucin 2 expression in colorectal cancer patients. A: Correlation between tissue mucin 2 (MUC2) expression and disease-free survival in colorectal cancer (CRC) patients; B: Correlation between tissue MUC2 expression and overall survival in CRC patients; C: Histogram of the recurrence rate of CRC patients with different tissue levels of MUC2 expression. DFS: Disease-free survival; OS: Overall survival. MUC2: Mucin 2; CRC: Colorectal cancer.

DISCUSSION

The protein encoded by the *MUC2* gene is the most abundant secreted mucin, covering the surface of the intestinal mucosa in the form of a gel and forming the skeleton of the mucus layer, protecting the intestine in many ways[16]. Recently, Javitt *et al*[17] presented an integrated structural analysis of the intestinal mucin MUC2, and revealed that the mucin assembly mechanism and its adaptation for hemostasis provide the foundation for rational manipulation of barrier function and coagulation[17]. On the other hand, CRC shows multiple complex pathologies based on the impaired structure and function of the intestinal mucosal barrier, and is associated with the disordered expression and dysfunction of mucins[18]. However, controversial findings of MUC2 function in the occurrence and development of CRC have required further invest-

igation to uncover the underlying mechanisms. We showed that MUC2 expression was decreased in carcinomas, compared with adjacent normal tissues, but CRC patients had higher serum levels of MUC2 compared with normal controls.

MUC2 plays an important role in maintaining the homeostasis of the intestinal environment and protecting susceptible bacteria from pathogenic microorganisms and/or toxic substances[19]. We detected decreased MUC2 in CRC tissue, compared to adjacent intestinal tissues, which may be related to the suppressed immune function in intestinal homeostasis. Importantly, decreased MUC2 expression was found to be associated with advanced CRC stage, suggesting a tumor-suppressive role in the development of CRC. In CRC patients with lymph node metastasis, the tissue MUC2 level was also lower than that in CRC patients without lymph node metastasis, further suggesting a potential protective role of MUC2 in lymph node metastasis in CRC patients. Although statistical significance was not found in the relationship between tissue MUC2 expression and tumor size, depth of invasion, or degree of differentiation, our results indicate that a more severe malignant biological behavior was more likely found in CRC tissues with low MUC2 expression, demonstrating a potential protective role of MUC2 in the development of CRC.

The expression of MUC2 in CRC tissues is reported to be related to the histopathological types of CRC. The expression of MUC2 in mucinous adenocarcinoma is increased, while that in non-mucinous adenocarcinoma is decreased[8,20]. In this study, patients with mucinous adenocarcinoma tended to have a higher proportion of patients with high expression of MUC2 than those with non-mucinous adenocarcinoma. However, the underlying roles of MUC2 in different types of CRC are still unclear and need further investigation.

Li *et al*[21] reviewed the prognostic and clinicopathological significance of MUCs in CRC, and demonstrated that upregulated MUC2 expression is associated with a better OS, while upregulated MUC1 expression is associated with a poor OS[21]. Elzagheid *et al*[22] reported that loss of MUC2 expression is associated with disease recurrence and tumor location. However, in multivariate survival analysis, MUC2 lost its power as an independent predictor of DFS and disease-specific survival[22]. To verify the prognostic value of MUC2 expression in CRC patients, we used Kaplan-Meier curve analyses and showed that low expression of tissue MUC2 was associated with a poor DFS and OS in CRC patients, which may be related to the high recurrence rate exhibited by CRC patients with low tissue MUC2 levels.

To investigate the potential function of secreted MUC2 in CRC patients, this study further analyzed the expression of serum MUC2 in CRC patients and normal controls. As serum DAO and D-LAC are biomarkers of the functional status of the intestinal mucosal barrier[23], we also monitored serum DAO and D-LAC levels. Interestingly, the serum level of MUC2 was positively related with serum DAO and D-LAC levels, which indicates dysfunction of intestinal mucosal barriers and increased intestinal permeability in CRC patients. The increased serum level of MUC2 may be associated with the progress of tumor infiltration.

The increased serum levels of MUC2 may be related to the fact that cancer cells gradually destroy the mucus layer, intestinal mucosal epithelial cells, and tight junctions, which result in the destruction of the MUC2 skeletal structure in the mucus layer, the apoptosis of intestinal mucosal epithelial cells, and abnormal expression and distribution of tight junction proteins. Decreased MUC2 in the intestinal mucosa and the damage of the intestinal barrier could result in invasion of various pathogenic microorganisms and toxic substances in the intestinal cavity to further aggravate the damage of the intestinal barrier and constitute a vicious circle, increasing intestinal mucosal permeability and promoting the translocation of MUC2 from epithelial cells to the blood.

CONCLUSION

This study found that MUC2 in intestinal tissues may play a protective role in the intestine and can be used as one of the indicators to evaluate the prognosis of patients with CRC. When the intestinal mucosal barrier function of patients with CRC is impaired, the serum level of MUC2 can reflect the severity of damage. Subsequent studies can further investigate the role of MUC2 in the malignant transformation of colorectal inflammatory diseases, cancer cell proliferation, invasion, metastasis, and the mechanism of resistance to chemotherapeutic drugs at the molecular level.

ARTICLE HIGHLIGHTS

Research background

At present, several studies have reported abnormal expression patterns of mucin 2 (MUC2) in cancerous lesions, including colorectal cancer (CRC). However, as a member of the intestinal mucosal mechanical barrier, the relationship between MUC2 and the intestinal mucosal barrier in patients with CRC remains unclear. Revealing this association will help us more fully understand the role of MUC2 in CRC.

Research motivation

Although many studies have proved that intestinal mucosal barrier function is impaired and MUC2 expression is abnormal in patients with CRC, the direct relationship between MUC2 and intestinal mucosal barrier has rarely been studied. The main problem to be solved in this study is to clarify this relationship and lay a foundation for further studies on the molecular mechanism of MUC2 involvement in CRC.

Research objectives

This study aimed to explore abnormal expression patterns of MUC2 and the relationship between MUC2 and intestinal mucosal barrier by characterizing the multiple expression patterns of MUC2 in CRC. The findings will provide a basis for further study of the pathogenesis of MUC2 in the process of intestinal mucosal barrier damage in CRC.

Research methods

Immunohistochemical staining was performed on cancer tissue and normal tissue samples from 100 patients with CRC to evaluate the expression of MUC2 in two different tissues, and these patients were followed for 12-60 mo to understand the overall survival (OS) and disease-free survival (DFS). Preoperative serum levels of MUC2, diamine oxide (DAO), and D-lactate (D-LAC) in 66 patients with CRC were detected by enzyme-linked immunosorbent assay and compared with those in 20 normal controls, so as to evaluate the damage of intestinal mucosal barrier in patients with CRC. The statistical methods involved in this study include χ^2 test, Fisher's exact test, Kaplan-Meier curve, and log-rank tests.

Research results

Immunohistochemical staining results showed that the expression of MUC2 in cancer tissues was lower than that in normal tissues (54% *vs* 79%, $P < 0.05$), and the expression of MUC2 was correlated with tumor-node-metastasis (TNM) stage and lymph node metastasis in CRC patients ($P < 0.05$), but not significantly related to the patient's age, sex, tumor location, size, depth of invasion, or degree of differentiation. The serum levels of MUC2, DAO, and D-LAC in patients with CRC were higher than those in normal people ($P < 0.05$), and were positively associated with serum levels of human DAO ($\chi^2 = 3.957$, $P < 0.05$) and D-LAC ($\chi^2 = 7.236$, $P < 0.05$), which are the biomarkers of the functional status of the intestinal mucosal barriers. It was suggested that the intestinal mucosal barrier was damaged, and MUC2 can also be used as a new evaluation index. The serum levels of MUC2 were correlated with TNM stage, tumor type, and distant metastasis in CRC patients ($P < 0.05$). It seems to be a trend that patients with higher malignancy and later stage of tumors have higher serum MUC2 levels. Survival analysis showed that decreased expression of MUC2 in CRC tissues predicted a poor survival. The expression of MUC2 in tissues was significantly correlated with DFS ($P = 0.032$) and OS ($P = 0.037$). And the recurrence rate of patients with low expression of MUC2 was higher than that of patients with high expression of MUC2 (40% *vs* 18.5%, $\chi^2 = 5.485$, $P < 0.05$).

Research conclusions

MUC2 in the intestinal tissue may play a protective role on the intestine, which can be used as an indicator to evaluate the prognosis of CRC patients. Intestinal mucosal barrier function of CRC patients is impaired, and the serum MUC2 level can reflect the severity of the damage.

Research perspectives

Future researchers can further study the molecular mechanism of MUC2 in the process of intestinal mucosal barrier damage, which may reveal the pathological mechanism of CRC from a new perspective and provide a basis for the development of new targeted

therapy drugs. In addition, related research can also be carried out in inflammatory bowel disease.

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ORIGINAL ARTICLE

Retrospective Study

Clinical characteristics of patients in their forties who underwent surgical resection for colorectal cancer in Korea

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Author contributions: Lee CS curated data and wrote this manuscript; Kwak JM, Kim J and Kim SH reviewed the paper; Baek SJ mainly provided the main conceptualization.

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Abstract

BACKGROUND

The proportion of young patients with colorectal cancer (CRC), especially in their 40s, is increasing worldwide.

AIM

To confirm the clinical characteristics of such patients, we planned a study comparing them to patients in their 30s and 50s.

METHODS

Patients undergoing primary resection for CRC, patients in their 30s, 40s and 50s were included in the study. Patient and tumor characteristics, and perioperative and oncologic outcomes were compared.

RESULTS

Most clinical characteristics of 451 (10.5%) patients in their 40s were more similar to those of patients in their 30s than those in their 50s. On pathology data, there were more metastatic lesions (30s *vs* 40s *vs* 50s; 17.5% *vs* 21.1% *vs* 14.9%, $P = 0.012$) in patients in their 40s. There was a trend toward less frequent K-ras mutations among patients in their 40s (48.5% *vs* 33.3% *vs* 44.5%, $P = 0.064$). The proportion of patients receiving postoperative chemotherapy was also significantly greater among patients in their 40s (58.3% *vs* 63.9% *vs* 56.3%, $P = 0.032$). Five-year overall survival (OS) and disease-free survival (DFS) did not differ between the three groups (5-year OS, 92.2% *vs* 89.8% *vs* 92.2%, $P = 0.804$; 5-year total DFS, 98.6% *vs* 95.7% *vs* 96.1%, $P = 0.754$; 5-year local DFS, 98.6% *vs* 94.3% *vs* 94.9%, $P = 0.579$; 5-year systemic DFS, 86.4% *vs* 87.9 % *vs* 86.4%, $P = 0.908$).

CONCLUSION

Patients with CRC in their 40s showed significantly more numerous metastatic lesions. The oncologic outcome of stage 1-3 patients in their 40s was not inferior compared to that of those in their 30s and 50s.

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Core Tip: The age at which colorectal cancer is first diagnosed is decreasing worldwide, and colorectal cancer, especially in the 40s is very important. In our study, we found that colorectal cancer patients in their 40s had significantly more metastatic lesions and fewer K-ras mutations. Nevertheless, the oncologic outcome was never inferior compared to patients in their 30s and 50s by stages. We believe this to be a very important clinical message in the current situation.

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INTRODUCTION

Because colorectal cancer (CRC) is the third most common cancer in the world and the second most common cancer in Korea, it is a health-sociologically important cancer. The epidemiology of CRC (incidence, age distribution of CRC, *etc.*) varies greatly among different countries and races. In Korea, the proportion of CRC patients under the age of 50 is among the highest in the world. Based on the annual report of cancer statistics in Korea, of the 25881 patients diagnosed with newly developed CRC in 2019, about 10% were patients under 50[1]. Not only is the proportion of young patients high in Korea, but the rate of increase is also very rapid both in Korea and worldwide [2-7]. In the United States, data gathered over the past 40 years confirms the proportion of young CRC patients has increased gradually, mainly among patients in their 40s, and especially among African-American and Hispanic patients[8,9]. A previous study explained that the recent increase in young colorectal cancer patients is related with changes to Western style diet, in lifestyle, and an increase in environmental carcinogens[10].

Amid the global trend of increasing proportion and importance of young patients with CRC, clinical characterization of CRC patients in their 40s is somewhat unique. Patients with colorectal cancer in their 40s have the potential for both late-onset hereditary CRC and early-onset sporadic CRC. Patients in their 40s have lived long enough to be exposed to environmental carcinogens and they are in the age group where sporadic CRC can develop. However, the possibility of hereditary CRC cannot be excluded in this group, as it is relatively young compared to the average age of CRC. In hereditary CRC, genetic counseling, including somatic mutation testing, is important. However, in countries with many young CRC patients, such as Korea, genetic counseling for all patients in their 40s is not available and is not cost effective. In addition, there are secondary problems that are sometimes overlooked even in patients who need to be examined genetic testing[11].

Whatever the cause, the recent rapid increase in number of young CRC patients is a socially important issue. In particular, young people have an important socioeconomic role, so the increase in young CRC patients is directly linked to socioeconomic problems. Therefore, it is very important to understand and actively manage the characteristics of these patients. However, few studies have analyzed the characteristics of patients with CRC in their forties[12-14]. Therefore, we planned to determine the proportion of patients in their 40s with CRC and to identify their clinical characteristics, especially compared with those in their 30s and 50s, and to develop a management plan accordingly.

MATERIALS AND METHODS

Patients and study design

Among the 4326 patients with CRC who underwent primary resection in our hospital from September 2006 to July 2019, patients in their 30s, 40s, and 50s were included in the study. Only primary pathologically confirmed adenocarcinomas located in the area from the appendix to the rectum were included in the study. Patients who had only diversion without resection, or who had recurrent cancer or metastases of other cancers were excluded from the study. This study was approved by the Institutional Review Board of Korea University Anam Hospital (No. 2020AN0309) and all participants provided informed consent.

Surgical data processing

In this study, cancers of the appendix, cecum, and ascending, hepatic flexure, and transverse colon were classified as right-sided colon cancer, while cancers of the splenic flexure, descending, and sigmoid colon were classified as left-sided colon cancer, and cancers of the rectosigmoid colon were classified as rectal cancer. In our institution, most surgeries have been performed minimally invasively since 2006. In addition, D3 Lymphadenectomy is routinely performed in radical operations for all CRCs. The types of surgical procedure were so diverse that they were classified into lesional radical operations (including complete mesocolic excision or total mesorectal excision), total surgery (total abdominal colectomy or total proctocolectomy), and limited surgery. In rare cases, two or more segmental colectomies were performed simultaneously.

Perioperative management and surveillance

Our institution conducts perioperative evaluation and treatment based on the National Comprehensive Cancer Network guidelines. All patients with colon cancer had been evaluated preoperatively by physical examination, total colonoscopy, abdominopelvic computed tomography (CT), chest CT, and routine laboratory testing, which included tests for tumor markers. If necessary, additional tests, such as rectal magnetic resonance imaging (MRI), liver MRI, and positron emission tomography-CT, were performed. If necessary, stent insertion or neoadjuvant chemotherapy or radiotherapy were performed before surgery. When surgery was performed in an emergency, studies that were not evaluated before surgery were performed as soon as possible after surgery. If there were no problems after surgery, water intake was allowed the day after surgery and a soft diet started on the second day after surgery. After surgery, adjuvant treatment was performed as appropriate considering the stage of cancer, the patient's age, general condition, and socioeconomic status. After adjuvant treatment, follow-up examinations were carried out at 3-mo intervals during the first 2 years postoperatively, at 6-mo intervals until 5 years after surgery, and then annually if there was no evidence of recurrence.

Statistical analysis

Patients were divided into three groups comprising patients in their 30s, 40s, or 50s to compare patient and tumor characteristics, perioperative data, and oncologic outcomes. Descriptive results were presented as the mean or median for continuous outcomes and as frequency or percentage for categorical outcomes. For comparison between two groups, Student's *t*-tests were used to compare continuous variables, and the χ^2 test or Fisher's exact test was applied for categorical variables. ANOVA was used to compare the three groups. Five-year overall survival (OS) and disease-free survival (DFS) were analyzed using the Kaplan-Meier method. Statistical analyses was performed using SPSS® version 22.0 (IBM, Armonk, NY, United States). A P value < 0.05 was considered statistically significant.

RESULTS

Of the 4326 patients included in the study, 120 were in their 30s (2.8%), 451 were in their 40s (10.5%), and 1044 were in their 50s (24.4%). The proportion of patients in their 40s did not significantly increase or decrease over time (Figure 1). Compared to those in their 30s and 40s, the proportion of men among patients in their 50s was greater (55.0% *vs* 56.8% *vs* 65.7%, P = 0.001) (Table 1). There were significant co-morbidities among patients in their 50s, especially cardiovascular diseases (2.5% *vs* 11.8% *vs* 29.8%,

Table 1 Patient characteristics

	30s (*n* = 120)	40s (*n* = 451)	50s (*n* = 1044)	*P* value[1]	*P* value[2]	*P* value[3]	*P* value[4]
Sex, male (%)	55.0	56.8	65.7	0.730	0.001	0.020	0.001
Co-morbidity (%)	28.3	37.3	52.9	0.070	< 0.001	< 0.001	< 0.001
Endocrine	3.3	9.5	14.3	0.028	0.012	0.001	< 0.001
CVD	2.5	11.8	29.8	0.002	< 0.001	< 0.001	< 0.001
Pulmonary	5.8	3.5	6.3	0.259	0.031	0.835	0.096
Other	20.8	21.1	20.6	0.956	0.837	0.951	0.979
ASA score (%)				0.079	< 0.001	< 0.001	< 0.001
1	47.5	40.8	30.4				
2	50.8	56.3	66.3				
3	0	2.0	2.4				
4	0	0.2	0.1				
5	0	0	0.1				
Unknown	0.8	0.7	0.8				
BMI, mean (kg/m^2)	23.2	23.3	23.7	0.899	0.023	0.143	0.049
Tumor location (%)				0.975	0.061	0.268	0.126
Right-sided colon	20.0	16.0	20.9				
Left-sided colon	21.7	29.0	27.7				
Rectum	55.8	53.4	50.0				
Multiple	2.5	1.6	1.5				
CEA, median (ng/mL)	1.8	2.5	2.1	0.497	0.615	0.190	0.462
CA, median 19-9 (IU/mL)	11.1	12.6	11.3	0.215	0.527	0.887	0.781
Neoadjuvant treatment (%)	26.7	25.5	15.3	0.795	< 0.001	0.002	< 0.001
Preoperative complication (%)	14.2	17.3	12.9	0.678	0.910	0.637	0.883
Treatment	3.3	7.8	5.8	0.378	0.327	0.706	0.517

[1]30s *vs* 40s.
[2]40s *vs* 50s.
[3]30s *vs* 50s.
[4]30s *vs* 40s *vs* 50s. CVD: Cardiovascular disease; ASA: American Society of Anesthesiologists; BMI: Body mass index; CEA: Carcinoembryonic antigen.

$P < 0.001$), and endocrine disorders, including diabetes mellitus (3.3% *vs* 9.5% *vs* 14.3%, $P < 0.001$), and the American Society of Anesthesiologists score was also high ($P < 0.001$). Body mass index was also greater among patients in their 50s (23.2 *vs* 23.3 *vs* 23.7 kg/m^2, $P = 0.049$). Tumor location, preoperative carcinoembryonic antigen, carbohydrate antigen 19-9 Level, preoperative complications and treatment did not differ between the three groups, but more neoadjuvant treatments were performed in patients in their 30s and 40s (26.7% *vs* 25.5% *vs* 15.3%, $P < 0.001$).

The rate of emergency surgery tended to be higher in the younger age group, but the difference was not statistically significant (5.0% *vs* 2.4% *vs* 1.8%, $P = 0.077$; Table 2). Patients in their 50s had fewer open surgeries and more minimally invasive surgery than those in their 30s or 40s ($P = 0.043$). Operative procedures, placement of permanent colostomy, operation time, and estimated blood loss were similar in the three groups, but the combined operation rate was greater among patients in their 30s and 40s than those in their 50s (15.0% *vs* 15.7% *vs* 10.7%, $P = 0.021$). Postoperative pathology outcomes did not differ between the three groups in terms of T stage, N stage, number of positive lymph nodes (LNs), tumor size, proximal resection margin, distal resection margin, and circumferential resection margin, but the number of LNs retrieved from patients in their 30s was greater than from patients in their 40s or 50s (34 *vs* 29 *vs* 27, $P < 0.001$) (Table 3). Patients in their 40s, unlike other groups, had many

Table 2 Operative data

	30s (*n* = 120)	40s (*n* = 451)	50s (*n* = 1044)	*P* value[1]	*P* value[2]	*P* value[3]	*P* value[4]
Emergency (%)	5.0	2.4	1.8	0.143	0.434	0.023	0.077
Operation type (%)				0.931	0.023	0.155	0.043
Laparoscopy	55.8	59.2	70.1				
Robot	35.8	31.2	21.6				
Open	5.8	6.2	4.0				
Conversion	1.7	1.5	2.0				
Transanal	0.8	1.8	2.2				
Operation procedure (%)				0.847	0.914	0.897	0.983
Lesional (CME, TME)	93.3	95.6	96.1				
Total (TAC, TPC)	5.0	1.8	1.1				
Multiple	0	0.7	0.3				
Limited (Segmental, TAE, TAMIS)	1.7	2.0	2.6				
Combined operation (%)	15	15.7	10.7	0.835	0.008	0.172	0.021
Permanent colostomy (%)	3.3	4.9	3.1	0.472	0.085	0.872	0.222
Operative time, median (min)	200	205	190	0.476	0.026	0.571	0.078
EBL, mean (mL)	69.4	92.9	100.0	0.331	0.685	0.316	0.555

[1]30s *vs* 40s.
[2]40s *vs* 50s.
[3]30s *vs* 50s.
[4]30s *vs* 40s *vs* 50s. CME: Complete mesocolic excision; TME: Total mesorectal excision; TAC: Total abdominal colectomy; TPC: Total proctocolectomy; TAE: Transanal excision; TAMIS: Transanal minimally invasive surgery; EBL: Estimated blood loss.

metastatic lesions (17.5% *vs* 21.1% *vs* 14.9%, $P = 0.012$), and the TNM stage was high ($P = 0.002$). Tumor differentiation and venous/lymphatic invasion were not different among the three groups, and there were no significant differences in immunohistochemistry (IHC) or molecular pathology test results. In the younger age group, mucinous type cancers, perineural invasion, and *BRAF* positivity were more frequent, but these findings were not statistically significant (7.5% *vs* 5.1% *vs* 4.7%, $P = 0.347$; 13.3% *vs* 9.5% *vs* 7.9%, $P = 0.094$; 15.8% *vs* 4.8% *vs* 3.9%, $P = 0.076$). Similarly, a greater proportion of microsatellite instability (MSI)-high (H) was found in the younger age group (16.7% *vs* 9.0% *vs* 3.7%, $P = 0.046$). while K-ras mutation was detected less frequently in patients in their 40s (48.5% *vs* 33.3% *vs* 44.5%, $P = 0.076$).

The postoperative course was generally similar among the three groups, and there were no differences in postoperative complications or length of stay. However, the enforcement rate of postoperative chemotherapy was significantly greater in the patients in their 40s (58.3% *vs* 63.9% *vs* 56.3%, $P = 0.032$) (Table 4). When comparing survival after excluding stage 4 patients, 5-year overall survival was 92.2% among patients in their 30s, 89.8% among patients in their 40s, and 92.2% among patients in their 50s, and there were no differences between the three groups ($P = 0.804$) (Figure 2). There were also no differences in 5-year disease-free survival between the three groups (total, 98.6% *vs* 95.7% *vs* 96.1%, $P = 0.754$; local, 98.6% *vs* 94.3% *vs* 94.9%, $P = 0.579$; systemic, 86.4% *vs* 87.9% *vs* 86.4%, $P = 0.908$).

DISCUSSION

In our study, there were relatively fewer K-ras mutations in CRC samples obtained from patients in their 40s than in those of the other age groups. Patients in their 40s had aggressive CRC at a high stage and were administered active perioperative treatment. As a result, the oncologic outcome of patients in their 40s excluding stage 4 patients was not worse than that of those in their 30s and 50s.

Table 3 Pathology data

	30s (*n* = 120)	40s (*n* = 451)	50s (*n* = 1044)	*P* value[1]	*P* value[2]	*P* value[3]	*P* value[4]
pT (%)				0.251	0.986	0.214	0.446
Tis	6.7	5.8)	8.1				
T0	5.0	4.0	4.0				
T1	9.2	6.4	10.8				
T2	12.5	12.0	13.6				
T3	55.0	62.5	54.8				
T4	11.7	9.3	8.3				
Unknown	0	0	0.3				
pN (%)				0.419	0.289	0.831	0.521
N0	56.7	50.8	57.2				
N1	24.2	28.2	25.3				
N2	16.7	17.7	14.1				
Unknown	2.5	3.3	3.4				
Positive LN, mean (*n*)	1.9	1.8	1.8	0.709	0.960	0.803	0.958
Retrieved LN, mean (*n*)	34	29	27	0.030	0.015	< 0.001	< 0.001
M (%)				0.438	0.003	0.386	0.012
M0	81.7	78.3	84.8				
M1	17.5	21.1	14.9				
Unknown	0.8	0.7	0.3				
Stage (%)				0.138	< 0.001	0.613	0.002
0	11.7	8.6	11.3				
1	15.8	13.1	18.6				
2	28.3	27.1	25.7				
3	26.7	29.3	28.3				
4	17.5	21.1	14.8				
Unknown	0	0.9	1.3				
Tumor size, mean (cm)	4.9	4.7	4.3	0.797	0.079	0.207	0.125
PRM, mean (cm)	19.2	16.6	16.3	0.044	0.699	0.395	0.530
DRM, mean (cm)	6.7	6.0	6.8	0.431	0.205	0.690	0.412
CRM, mean (cm)	0.6	0.7	0.7	0.856	0.834	0.681	0.927
Differentiation (%)				0.279	0.670	0.155	0.347
Well	22.5	17.5	18.6				
Moderate	56.7	68.3	67.0				
Poor	5.0	2.9	2.4				
Mucinous	7.5	5.1	4.7				
Signet ring cell	0.8	0	0.3				
Etc.	0.8	0	0.1				
Unknown	6.7	6.2	6.9				
Venous invasion (%)	8.3	5.1	4.9	0.159	0.893	0.101	0.254
Lymphatic invasion (%)	20.0	15.3	16.8	0.194	0.432	0.365	0.415
Perineural invasion (%)	13.3	9.5	7.9	0.199	0.297	0.036	0.094

IHC (% positive)							
EGFR	53/63 (84.1)	210/274 (76.6)	506/673 (75.2)	0.197	0.636	0.113	0.275
CDX-2	23/24 (95.8)	84/87 (96.6)	236/239 (98.7)	0.869	0.194	0.268	0.338
P53	50/52 (96.2)	217/223 (97.3)	549/572 (96.0)	0.657	0.369	0.951	0.668
MLH-1	31/33 (93.9)	162/165 (98.2)	398/407 (97.8)	0.158	0.767	0.174	0.326
MSH-2	33/33 (100)	165/165 (100)	403/408 (98.8)	-	0.154	0.524	0.295
MSH-6	24/24 (100)	118/120 (98.3)	292/295 (98.9)	0.528	0.583	0.621	0.738
PMS-2	19/20 (95)	97/100 (97)	240/249 (96.4)	0.652	0.777	0.754	0.899
BRAF	3/19 (15.8)	3/63 (4.8)	7/180 (3.9)	0.108	0.765	0.024	0.076
Molecular test (% wild-type)							
K-ras	17/33 (51.5)	92/138 (66.7)	152/274 (55.5)	0.105	0.029	0.667	0.064
N-ras	20/21 (95.2)	90/95 (94.7)	193/200 (96.5)	0.926	0.475	0.770	0.768
Braf	12/12 (100)	51/53 (96.2)	93/97 (95.9)	0.502	0.917	0.478	0.778
MSI							
MSS	14/18 (77.8)	59/67 (88.1)	142/163 (87.1)	0.271	0.846	0.278	0.506
MSI-L	1/18 (5.6)	2/67 (3.0)	15/163 (9.2)	0.605	0.102	0.607	0.248
MSI-H	3/18 (16.7)	6/67 (9.0)	6/163 (3.7)	0.351	0.103	0.016	0.046

[1]30s *vs* 40s.

[2]40s *vs* 50s.

[3]30s *vs* 50s.

[4]30s *vs* 40s *vs* 50s. LN: Lymph node; PRM: Proximal resection margin; DRM: Distal resection margin; CRM: Circumferential resection margin; IHC: Immunohistochemistry; EGFR: Epidermal growth factor receptor; MSI: Microsatellite instability.

CRC in patients in their 40s may represent either late onset hereditary cancer or early onset sporadic cancer. While genetic factors are the major cause of CRC in patients under the age of 40, patients in their 40s have had relatively long periods of exposure to environmental factors, so the importance of environmental factors is relatively high. Therefore, the 40s are considered to be the age at which sporadic cancer, which occurs mainly in people over 50, begins. The distinction between hereditary and sporadic CRC has clinical significance in determining the scope of surgery, surveillance, and prevention of cancer occurrence in the family in advance [15]. Representative hereditary CRCs include familial adenomatous polyposis (FAP) and hereditary nonpolyposis CRC (HNPCC), which are characterized by onset at a young age, frequent synchronous and metachronous colorectal malignancies, and multiple extracolonic malignancies. The most important diagnostic criterion for hereditary CRC is the age of the patient; however, the appropriate cutoff age is unclear, given HNPCC (also called Lynch syndrome) typically occurs later than FAP, and it can therefore be difficult to identify sporadic CRC that developed early based only upon the patient's age. The Amsterdam criteria or Bethesda guidelines recommend that HNPCC be suspected if the patient is 50 years old or younger and has some other manifestations. However, in a country such as Korea with a large proportion of CRC patients of relatively young age, especially those in their 40s, it may be not available to conduct genetic studies in all patients in their 40s with CRC. On the other hand, if hereditary CRC is not identified where present in these patients, they may miss the additional therapeutic benefits from genetic counseling.

It should be noted that, in recent years, the age of onset of CRC is not only gradually decreasing in Korea, but also worldwide. In statistical analyses based on SEER data, Siegel *et al*[2] reported that colon cancer incidence rates increased by 1.0% to 2.4% annually since the mid-1980s in patients aged 20 to 39 years and by 0.5% to 1.3% since the mid-1990s in patients aged 40 to 54 years. Similar trends have also been identified in other countries such as Canada and Australia, and because of this, in 2018 the American Cancer Society recommended that the screening age for CRC be lowered from 50 to 45 years[16]. Thus, the dilemma regarding how to appropriately manage CRC patients in their 40s identified in Korea may be occurring in other countries. In

Table 4 Postoperative data

	30s (*n* = 120)	40s (*n* = 451)	50s (*n* = 1044)	*P* value[1]	*P* value[2]	*P* value[3]	*P* value[4]
Gas, median (d)	2	2	2	0.217	0.261	0.408	0.322
Stool, median (d)	2	2	3	0.995	0.002	0.071	0.004
Feed, median (d)	2	2	2	0.569	0.655	0.736	0.827
Postoperative hospital stays, median (d)	8	8	8	0.545	0.748	0.656	0.834
Postoperative complication (%)	27.5	25.9	23.1	0.731	0.235	0.281	0.339
Leakage	5.0	6.0	6.6	0.681	0.652	0.497	0.747
Intraabdominal abscess	3.3	2.4	1.6	0.587	0.289	0.184	0.319
Wound infection	2.5	1.3	1.2	0.361	0.893	0.264	0.532
Ileus	11.7	10.4	8.4	0.695	0.218	0.235	0.299
Bleeding	2.5	0.7	1.0	0.080	0.576	0.128	0.194
Stoma related	0	0.4	0.2	0.466	0.387	0.632	0.569
Pulmonary	0.8	1.1	0.9	0.793	0.650	0.974	0.896
Cardiovascular	0	0	0.2	-	0.353	0.632	0.579
Nephrology	0	0.4	0.2	0.466	0.387	0.632	0.569
Voiding	0.8	0.4	0.8	0.600	0.482	0.937	0.768
Chyle	3.3	3.8	4.0	0.822	0.817	0.714	0.921
Other	3.3	2.4	2.4	0.587	0.959	0.533	0.821
Reoperation (%)	1.7	2.4	3.6	0.615	0.232	0.262	0.297
Postoperative mortality (%)	0	0.4	0.3	0.466	0.632	0.557	0.722
Adjuvant treatment (%)	60.8	66.3	58.1	0.370	0.002	0.389	0.007
Chemotherapy	58.3	63.9	56.3	0.357	0.009	0.618	0.032
Radiotherapy	2.5	2.4	1.8	0.946	0.429	0.580	0.679
Recurrence (%)	15.8	20.2	16.4	0.187	0.088	0.657	0.168
Local	0.8	2.2	2.4	0.328	0.835	0.273	0.548
Systemic	6.7	6.9	6.8	0.870	0.837	0.956	0.975
Progression	8.3	11.8	7.8	0.318	0.019	0.824	0.061
Death (%)	11.7	12.4	8.7	0.824	0.027	0.286	0.074
Follow-up duration, median(mo)	26.7	25.6	24.8	0.694	0.819	0.579	0.850

[1]30s *vs* 40s.
[2]40s *vs* 50s.
[3]30s *vs* 50s.
[4]30s *vs* 40s *vs* 50s.

this regard, it is clinically important to confirm the characteristics of CRC patients in their 40s compared to those in their 30s and 50s.

In our study, patients in their 40s with CRC had independent characteristics that were distinct from those in their 30s and 50s. The proportion of women was relatively high, co-morbidity was low, and combined operations were frequent. The characteristics of patients in their 40s with CRC tended to be similar to those of patients in their 30s. However, multiple lesions were not as frequent as in those in their 30s, which led to a lower rate of total colectomy, and a significantly lower number of retrieved LNs than in patients in their 30s, similar to the characteristics of patients in their 50s. Contrary to expectation, histological characteristics were not different by age group. In younger patients, there were more cancers of the mucinous type, and greater perineural invasion, *BRAF* positivity on IHC, and rate of MSI-H, but these findings were not statistically significant.

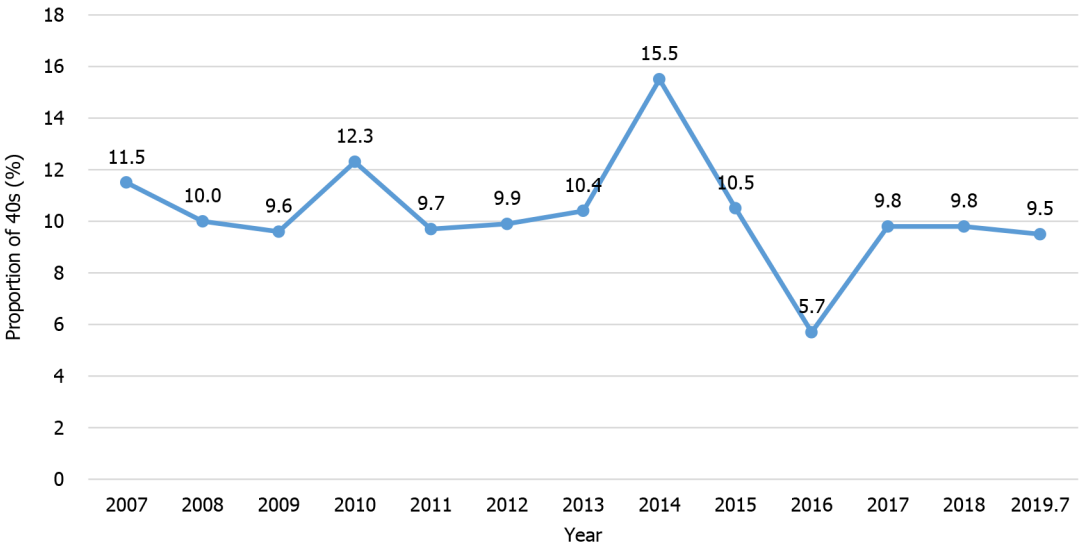


Figure 1 Changes in the proportion of colorectal cancer patients in their 40s by year.

It is noteworthy that in patients in their 40s, metastases were significantly more frequent at the time of diagnosis, as was pre-/post-surgery chemotherapy. Because many countries, including Korea, begin screening for CRC among average-risk people in their 50s, early-stage CRC in their 50s might be expected to be found relatively frequently by screening. Considering that the age is excluded from screening for CRC, it can be understood that the stage of CRC patients in their 40s is higher than those in their 50s. However, it is very surprising that the stage in the 40s is higher than even in the 30s. One hypothesis is that some of the patients with CRC in their 30s have been diagnosed with FAP or HNPCC by themselves or by their family members, and early CRC may have been detected in surveillance colonoscopy or prophylactically resected specimens. In our study, the frequency of K-ras mutation was significantly lower among patients in their 40s, but more research and consideration will be needed to determine the effect of mutation frequency on staging. Meanwhile, when assessing survival among Stage 1-3 patients, there were no differences between patients in their 40s and those in their 30s or 50s. We could find that the age of 40s itself is not a risk factor for poor survivals although other studies have been controversial about the results[12-14]. As a result, early detection of CRC patients in their 40s will be an important task. Given 54% of CRCs in patients in their 40s are rectal cancer and 28.7% are left-sided colon cancer, a new screening program in which sigmoidoscopy is recommended beginning in the 40s may be considered[17].

Another interesting finding from our study is that the proportion of CRC patients in their 40s did not change significantly throughout the study period, and has remained approximately 10% since 2007. This finding appears to conflict with those from Western countries, where the proportion of patients with CRC in their 40s has increased gradually over the last few decades[2-4]. In addition, the results of a recent analysis using the national registry of Asian countries are contrary to the reported trend in which the age at diagnosis of CRC is decreasing in several Asian countries, including Korea[5-7]. This inconsistency may have arisen because our study was conducted over a relatively short period of analysis. And, in our study, the percentage of CRC patients in their 40s was somewhat greater than in the annual report of cancer statistics in Korea, probably because our study included only patients who underwent surgery[1]. Given a significant proportion (about 10%) of all Korean CRC patients are in their 40s, an age group of major socioeconomic influence, there is an ongoing debate regarding reducing the age at which initiation of screening is recommended[16,18,19].

Our study had some limitations. First, the primary comparators were clinical variables, and immunohistochemistry tests such as epidermal growth factor receptor or CDX-2 were limited, and genetic tests such as *ras* gene or MSI were performed only in about 10%-50% of patients. In particular, in CRC cases in patients in their 30s, the likelihood of hereditary cancer is high, so it is typically necessary to perform genetic testing for somatic mutations, but there were few results. And, information on whether to diagnose FAP and HNPCC is also insufficient. The second limitation was that even in among patients in their 40s, the patient characteristics may differ between patients in their early and late 40s. Third, there were large differences in patient

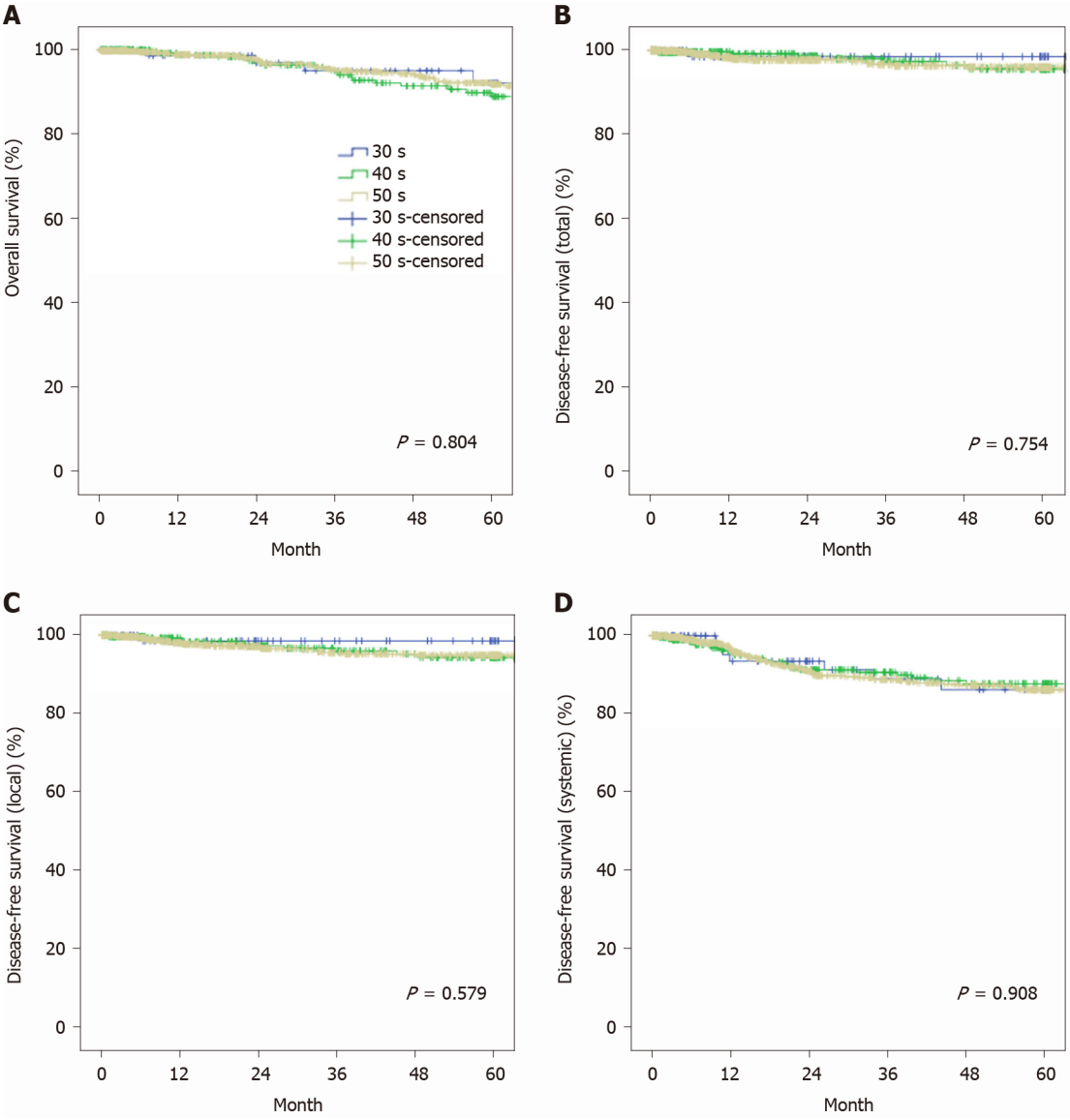


Figure 2 Comparison of 5-year overall survival, disease-free survival (total, local, and systemic) in stage 1-3 patients in their 30s, 40s and 50s with colorectal cancer. A: 5-year overall survival; B: Disease-free survival (total); C: Disease-free survival (local); D: Disease-free survival (systemic). OS: overall survival; DFS: disease-free survival.

numbers by age group, with 8-fold or more patients in their 50s than in their 30s. Despite these limitations, our findings can be meaningful in that identification of the characteristics of the growing proportion of CRC patients in their 40s may help to formulate patient management strategies.

CONCLUSION

In conclusion, patients with CRC in their 40s comprised about 10% of all CRC patients, and most of the tumor characteristics were similar to those in their 30s and 50s in our study. However, their cancers were often found at an aggressive stage compared to those found in the other age groups. Nonetheless, the oncologic outcome was not worse by stage, so it is necessary to actively perform screening tests for early detection. In addition, even in patients in their 40s, the potential for late-onset hereditary CRC must always be considered, but future studies are needed to establish new standards for conducting genetic tests for patients with CRC in their 40s.

ARTICLE HIGHLIGHTS

Research background

Globally, the proportion of young patients with colorectal cancer (CRC), especially in their 40s, is increasing, and this rate of increase is very rapid.

Research motivation

Patients with CRC in their 40s have the potential for both late-onset hereditary CRC and early-onset sporadic CRC. However, few studies have analyzed the characteristics of patients with CRC in their 40s.

Research objectives

The main aim of this study was to determine the proportion of patients with CRC in their 40s and to identify their clinical characteristics, especially compared with those in their 30s and 50s.

Research methods

We compared patient and tumor characteristics as well as perioperative outcomes of patients in their 30s, 40s, and 50s who received primary resection for CRC. In addition, the 5-year survivals were compared between the three groups, excluding stage 4.

Research results

In patients with CRC in their 40s, there were significantly more metastatic lesions and a higher TNM stage. K-ras mutations tended to be low in patients in their 40s, and postoperative chemotherapy was frequently performed in them. Excluding stage 4, the 5-year overall and disease-free survivals of CRC patients in their 40s did not differ from those in their 30s and 50s.

Research conclusions

Patients with CRC in their 40s exhibited relatively more metastatic lesions and a more advanced stage, but the oncologic outcomes of patients excluding stage 4 were not inferior compared to those in their 30s and 50s. Therefore, it is necessary to actively perform screening tests for early detection of CRC in those in their 40s.

Research perspectives

More analyses of immunohistochemistry or genetic testing results are needed to complement our results. In addition, future studies are also needed to establish new standards for conducting genetic tests for patients with CRC in their 40s.

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ORIGINAL ARTICLE

Observational Study

Effect of gastric microbiota on quadruple *Helicobacter pylori* eradication therapy containing bismuth

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Abstract

BACKGROUND

Helicobacter pylori (*H. pylori*) is an important pathogen that can cause a variety of diseases. Yet, full eradication of *H. pylori* remains a significant challenge in clinical practice. *H. pylori* and other microbial communities have complex interactions in the unique gastric microecological environment. However, it is not clear whether the interactions have any effect on the therapeutic effect of *H. pylori*.

AIM

The aim was to investigate the characteristics of the gastric microbiota with *H. pylori* infection and the influence on the *H. pylori* eradication treatment.

METHODS

Patients with *H. pylori* infection underwent gastroscopy and received treatment for eradication. The prescription included esomeprazole 20 mg bid, Livzon Dele 220 mg bid, amoxicillin 1000 mg bid, and clarithromycin 500 mg bid for 14 d. Patients who did not respond to treatment and failed eradication were compared with those who achieved eradication by 1:2 propensity matching. High-throughput sequencing of the gastric mucosal microbiota was performed, and the results were evaluated by alpha diversity analysis, beta diversity analysis, species correlation analysis, and metabolic pathway correlation analysis.

RESULTS

The eradication rate of all the patients was 95.5% (171/179). Twenty-four patients were enrolled in the study after propensity-matched scoring. There were eight cases in the failure group (patients who did not respond well to therapy) and 16 cases in the success group. The majority phyla in the two groups were the same, and included Proteobacteria, Bacteroides, Firmicutes, Actinomycetes, and

additional data are available.

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Fusobacteria. The microbial diversity in the failure group had a decreasing trend (P = 0.092) and the species abundance was significantly lower (P = 0.031) compared with the success group. The high rate of *H. pylori* eradication was associated with *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*, as they were significantly enriched in the successful group (P < 0.05). *Veronococcus* and *Cilium* were enriched in the mucosa of chronic atrophic gastritis patients compared with chronic superficial gastritis patients (P = 0.0466 and 0.0122, respectively). In both study groups, *H. pylori* was negatively correlated with other bacterial genera. More bacterial genera were directly related to *H. pylori* in the successful group compared with the failure group.

CONCLUSION

The effectiveness of quadruple *H. pylori* eradication therapy containing bismuth depended on gastric microbiota, and the high rate of *H. pylori* eradication was associated with the presence of *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*.

Key Words: *Helicobacter pylori*; Eradication; Quadruple therapy; Influence factors; propensity matching; Gastric microbiota

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Core Tip: *Helicobacter pylori* (*H. pylori*) is an important pathogen that can cause a variety of diseases. Its eradication can be affected by many factors. In this study, we explored the effect of the gastric microbiota on quadruple *H. pylori* eradication therapy containing bismuth. The results indicated that quadruple *H. pylori* eradication therapy containing bismuth was affected by the gastric microbiota. A high rate of *H. pylori* eradication was associated with the presence of *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*. Our findings may provide the basis for clinical treatment.

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INTRODUCTION

The gastric mucosal environment was long thought to be sterile. Yet, the discovery of *Helicobacter pylori* (*H. pylori*) put an end to the traditional view of the sterile stomach [1]. Furthermore, the development of modern technology led to a deeper understanding of gastric microbiota. Numerous gastric microbiota have been discovered over the years by 16S rRNA sequencing[2]. *H. pylori* and other microbial communities have complex interactions in the unique gastric microecological environment. *H. pylori* can inhibit other microbial communities by inducing the production of cytokines and antimicrobial peptides[3]. On the contrary, other microbial communities can affect *H. pylori*. For example, *Streptococcus* can change the spiral form of *H. pylori* into a spherical shape and inhibit its growth[4].

H. pylori is an important pathogen associated with a variety of diseases[5-7] including gastric cancer[8]. About 50% of the global population is infected with *H. pylori*[9]. *H. pylori* infection is an infectious disease and a "screening-treatment" strategy has been recommended for the treatment of *H. pylori*. However, the eradication of *H. pylori* can be affected by many factors, including antibiotic resistance[10], medication compliance[11,12], virulence factors[13], and others. In this study, we investigated the characteristics of the gastric microbiota in patients with *H. pylori* infection and the effect of the gastric microbiota on the success of quadruple *H. pylori* eradication therapy containing bismuth.

MATERIALS AND METHODS

Participants

Patients diagnosed with *H. pylori* infection in the Gastroenterology Department of Peking University Third Hospital between were enrolled between July 2018 and July 2019. Patients who were (1) 18-70 years of age; (2) with *H. pylori* infection confirmed by gastroscopy and histopathology were eligible. Patients (1) with previous *H. pylori* eradication therapy; (2) using proton pump inhibitors, H_2 receptor blockers, bismuth, antibiotics, or other drugs that might affect the study results within 4 wk of inclusion; (3) with gastrointestinal tumors; (4) with a history of gastric or esophageal surgery; (5) with Zollinger-Ellison syndrome; (6) with abnormal liver or kidney function; (7) with severe cardiovascular, respiratory, blood, endocrine, neurological, or mental disease; (8) with allergy to a study drug; (9) were pregnant or lactating; and (10) or with histories of alcohol abuse or clinical conditions that might increase the risk of side effects were excluded.

Methods

Before inclusion, all patients provided informed consent for clinical sample collection. A biopsy was taken from the antrum before *H. pylori* eradication treatment. A rapid urease test (RUT) was performed during the gastroscopy, and if the result was positive, gastric mucosa biopsies from the lesser curvature of antrum and corpus were collected, placed in a cryovial, and stored −80 ℃. At the same time, mucosa specimens were collected from the gastric antrum and corpus were collected for histopathological examination and Warthin-Starry (WS) staining.

Patients diagnosed with *H. pylori* infection by positive RUT results and WS staining were treated with esomeprazole 20 mg bid, amoxicillin 1000 mg bid, clarithromycin 500 mg bid, Livzon Dele bismuth potassium citrate 220 mg bid for14 d. A ^{13}C urease breath test (^{13}C-UBT) was performed 8 wk after treatment, which was considered successful if the ^{13}C-UBT was negative.

Patients were divided into failure and the success groups after their treatment was completed. Patients who did not respond well to therapy were included in the failure group. The success group was evaluated by nearest-neighbor matching, which is a type of propensity score matching and paired with patients in the failure group who had a similar propensity index. The propensity index was estimated by the model so as to equalize the covariates between the two groups. Gender, age, body-mass index (BMI kg/m^2), gastroscopy diagnosis, and background gastric mucosa were the covariables used to calculate the propensity values. Taking the sample size and matching quality, the allowable error was set to 0.1. The failure and success groups were matched at a ratio of 1:2. The gastric mucosa microbiota were assayed and compared according to the results of propensity score matching.

Microbial diversity sequencing

The total DNA of the microbiota was extracted following the instructions with E.Z.N.A.® soil DNA kits (Omega Bio-Tek, Norcross, GA, United States) following the manufacturer's instructions, and the quality of DNA extraction was assayed by 1% agarose gel electrophoresis. DNA concentration and purity were determined by a NanoDrop 2000 spectrophotometer, and 338 F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers were used for PCR amplification of the V3-V4 region of the 16S rRNA gene. The PCR products were recovered on 2% agarose gels after combining the PCR products of the same sample. Recovered products were purified with AxyPrep DNA gel extraction kits (Axygen Biosciences, Union City, CA, United States) and assayed with a Quantus™ Fluorometer (Promega, United States). The library was built with NEXTFLEX Rapid DNA-Seq Kits and sequenced with a Miseq PE300 platform (Illumina).

Sequence data analysis

Fastp software was used for quality control of the original sequence, and Flash software was used for stitching. Usearch software (version7.0, http://drive5.com/uparse/) was used to for operational taxonomic unit (OTU) clustering of sequences based on 97% similarity and elimination of chimeras. RDP classifier (http://rdp.cme.msu.edu/) was used to compare each sequence with the sequences included in Silva database (SSU132). The threshold was set to 70%, and the results of species classification annotations were obtained. The sample species composition was analyzed based on the annotation results.

The alpha diversity analysis (*i.e.* within-sample diversity) reflected species diversity, including Shannon, ace, and coverage indices. Beta diversity analysis (*i.e.* diversity between samples) or between-group differences in species composition was by principal coordinates analysis (PCoA). The Wilcoxon rank-sum test was used to compare the two groups. A P value of < 0.05 was considered significant. Correlation analysis of species was carried out by correlation heatmaps, network analysis, and metabolic pathway analysis. The relationship between the microbiota and the result of *H. pylori* eradication was preliminarily explained.

Statistical analysis

SPSS 23.0 was used for data analysis. measurement data were reported as means ± SD and compared by *t*-tests. Categorical data were compared by χ^2 tests P values of < 0.05 were considered statistically significant.

RESULTS

Basic information

Of the 179 enrolled patients, 171 responded well to therapy with successful *H. pylori* eradication. Eight patients failed treatment. The eradication therapy success rate was 95.5%. Propensity scoring resulted in matching eight failed cases with 16 successful cases. The results of gastroscopy revealed chronic gastritis in all patients; and after matching, there were no significant differences in the baseline characteristics between the two groups (Table 1).

A total of 1 204 878 reads and 1028 OTUs were obtained from 24 samples. The samples contained a mean of 50 203 reads and 191 OTUs. The reads in the failed eradication group (58 487) were significantly higher (P = 0.013) than those in the successful eradication group (46 061); the difference between the OTUs in the two groups was not significant (166 and 203, respectively, P = 0.719). The samples were randomly flattened according to the minimum number of sample sequences to avoid analysis deviation. A total of 980 OTUs were obtained, and each sample contained 30 043 reads. All samples were dominated by *H. pylori* at the genus level; *H. pylori* infection was pathologically confirmed.

Analysis of microbiota composition and differences

The proportions of bacterial species in the failure and the success groups were evaluated by Good's species coverage index, which found that the difference between the two groups was not significant (P = 0.125). The coverage index in both groups was higher than 0.99 and confirmed that the test results covered most bacterial species in the gastric mucosa. Analysis of community composition showed that the gastric mucosa microbiota mainly contained Proteobacteria, Bacteroidetes, Firmicutes, Actinomycetes, and Fusobacteria, regardless of the study group. The abundance of Proteobacteria was higher in the failure group than in the success group, and that of Actinobacteria was lower (Table 2).

Alpha and beta diversity reflect differences in the microbial composition. The results of the Shannon index, which is one an indexes of alpha diversity, showed that the diversity in the failure group was reduced compared with the success group, but the difference was not significant (P = 0.092; Figure 1A). The Ace index showed that the species abundance was significantly lower in the failure group than in the success group (P = 0.031; Figure 1B). The dominant species in both study samples was *H. pylori*, but the microbiota composition differed at the genus level (Figure 2A). PCoA analysis of beta diversity resulted in a weighted UniFrac showing that the total diversity of the first two principal coordinates was 89.96% and the difference between the two groups was significant (ANOSIM, P = 0.048; Figure 2B). According to the unweighted UniFrac, the difference between the two groups was significant (ANOSIM, P = 0.001; Figure 2C). Binary Euclidean analysis, which was used to assess the difference in species composition, showed a significant difference between the two groups (ANOSIM, P = 0.001; Figure 2D). As shown in Figure 3, *H. pylori* was more abundance in the failure than in the success group, but the difference was not significant (Wilcoxon rank-sum test, P = 0.0809). *Rhodococcus*, *Lactobacillus*, and *Sphingomonas* were significantly enriched in the success group, (Wilcoxon rank-sum test, P < 0.05).

The flora composition of gastric mucosa that were histologically different was also analyzed. The abundance of *H. pylori* was higher in the mucosa of chronic superficial gastritis but the difference was not significant (Wilcoxon rank-sum test, P = 0.1179).

Table 1 Baseline characteristics used in case matching of *Helicobacter pylori* eradication failure and success

		Failure group	Success group	*P* value
Sex	Male	7	14	1.000
	Female	1	2	
Age in yr, mean ± SD		40.13 ± 14.55	37.44 ± 6.42	0.532
BMI in kg/m^2, mean ± SD		25.01 ± 5.32	25.20 ± 2.84	0.913
Background mucosa	Chronic superficial gastritis	6	13	1.000
	Chronic atrophic gastritis	2	3	

SD: Standard deviation.

Table 2 Phylum-level differences of gastric mucosa microbiota

Phyla	Failure group, %	Success group, %	*P* value
Proteobacteria	98.26	94.60	0.0346
Bacteroidetes	0.60	1.23	0.4623
Firmicutes	0.52	1.23	0.2839
Actinomycetes	0.21	0.96	0.0016
Fusobacteria	0.11	0.27	0.6025

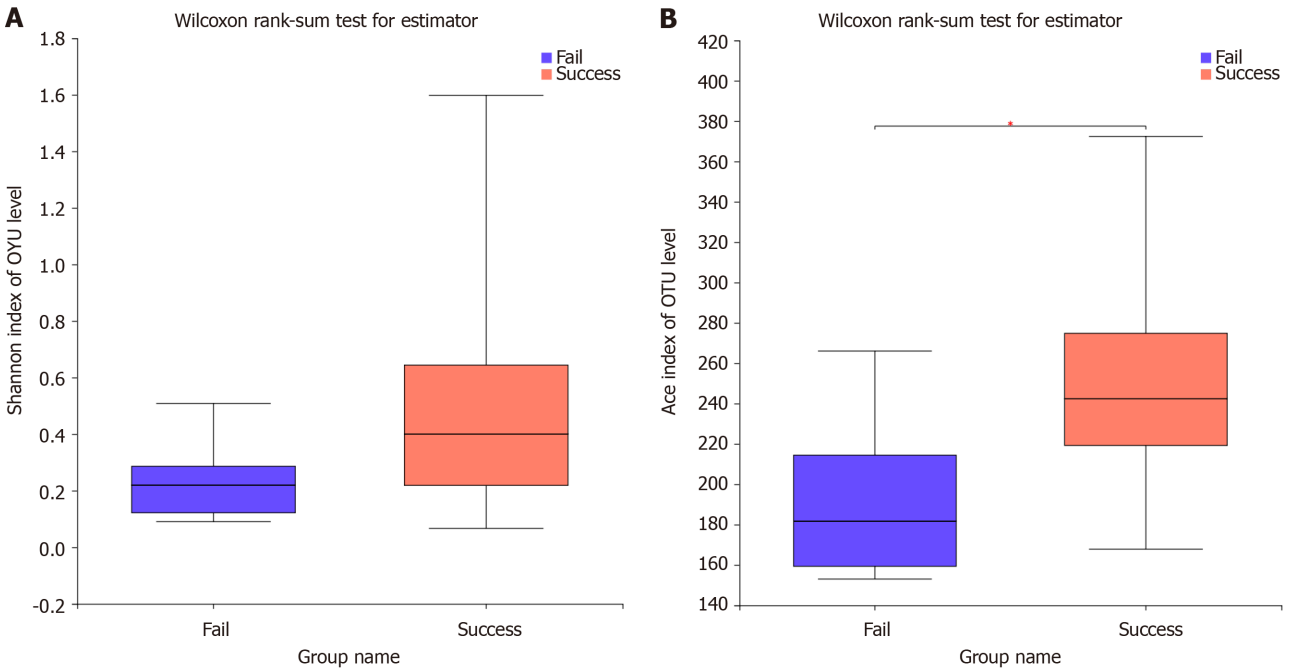


Figure 1 Alpha diversity analysis. A: Shannon index; B: Ace index.

Veronococcus and *Cilium* were more enriched in the mucosa of chronic atrophic gastritis (Wilcoxon rank-sum test, $P = 0.0466$ and 0.0122).

Correlation analysis of microbiota

Heatmap results of the species correlation analysis found that *H. pylori* was negatively correlated with other bacterial genera (Figure 4), *H. pylori* was negatively correlated with *Ralstonia* in the failure group (genus level) and negatively correlated with *Haemophilus, Prevotella, Streptococcus, Actinomycetes, Veillonella, Neisseria, Fusobacterium,* and *Leptotrichia* in the success group (genus level). The PICRUSt metabolic pathway

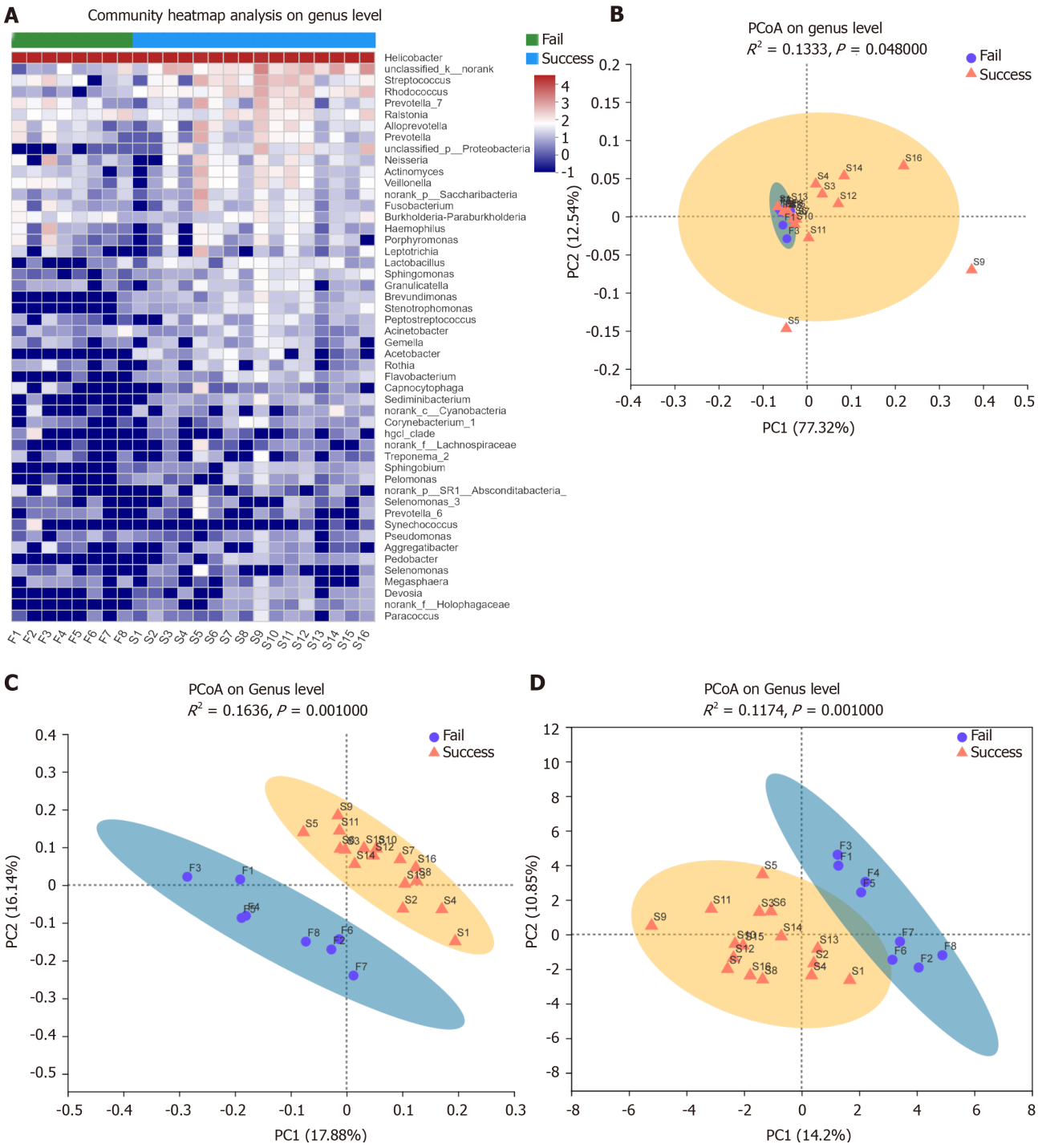


Figure 2 Beta diversity analysis. A: Heatmap; B: Weighted UniFrac; C: Unweighted UniFrac; D: Binary Euclidean. Points with different colors or shapes represent samples from different groups. The closer the two points are, the more similar the species composition is.

function prediction showed that the level two metabolic pathways in the two study groups were basically the same, mainly including carbohydrates, amino acids, energy, coenzyme factors, and vitamins.

DISCUSSION

The composition of the gastric mucosa microbiota infected by *H. pylori* mainly included Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, and Fusobacteria, which is consistent with the results reported by previous studies[14,15]. *H. pylori* infection can significantly reduce the diversity of gastric mucosal microbiota[16]. In this study, the Shannon index did not reveal a significant difference in the diversity of

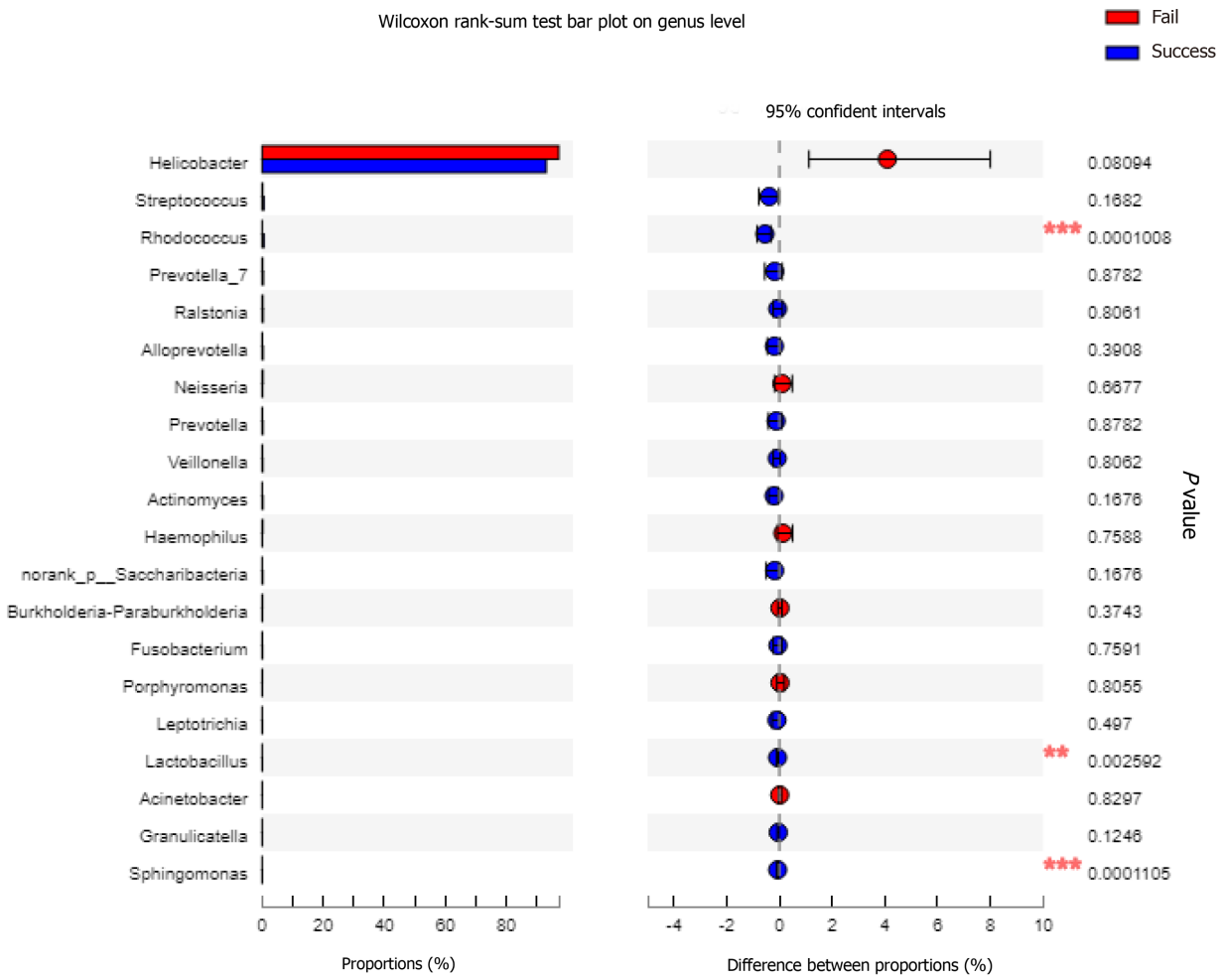


Figure 3 Genus-level differences between the success group and the failure group. The analysis includes the 20 most abundant genera.

the microbiota between the success and the failure group. However, the microbiota diversity in the failure group was lower, which suggests that eradication of *H. pylori* may be associated with the diversity of gastric mucosal microbiota.

The abundance of Proteobacteria was higher in the failure group. The genus level data suggests that the higher abundance of *H. pylori* in the eradication failure group might have caused an increased abundance of Proteobacteria. Although the difference in *H. pylori* abundance between the two groups was not significant, previous studies have shown that a higher abundance of *H. pylori* may reduce the effectiveness of empirical eradication therapy[17]. It has been reported that the abundance of *H. pylori* impacted only the result of traditional triple therapy[18]. Nevertheless, our results implied that the abundance of *H. pylori* had an impact on the eradication effect of quadruple therapy containing bismuth. In addition, 16s RNA may be more accurate than the $^{13}C/^{14}C$-UBT or histological evaluation for the determination of *H. pylori* abundance.

At the genus level, the gastric mucosa microbiota species diversity was similar in the two groups. However, PCoA analysis showed that there were differences in the species composition and abundance between the two groups. The Ace index confirmed the differences in abundance of the two groups, and statistical analysis confirmed that the success group was significantly enriched in *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*. The reason for the increased abundance of Actinomycetes in the success group may be related to the enrichment of *Rhodococcus*, an opportunistic pathogen[19,20] that can be detected in the stool of healthy people. Thiamine is required for the growth of *Rhodococcus*[21] and is essential nutrient for the growth of *H. pylori*[22]. *H. pylori* is a thiamine auxotroph that lacks the gene that synthesizes thiamine[23]. Vitamin metabolism is one of the microbiota's main metabolic pathways predicted by PICRUSt. Therefore, *Rhodococcus* may inhibit the growth of *H. pylori* through the acquisition of thiamine. *Lactobacilli*, which are beneficial bacteria, effectively improve *H. pylori* eradication if added to the prescription, especially in 7-d

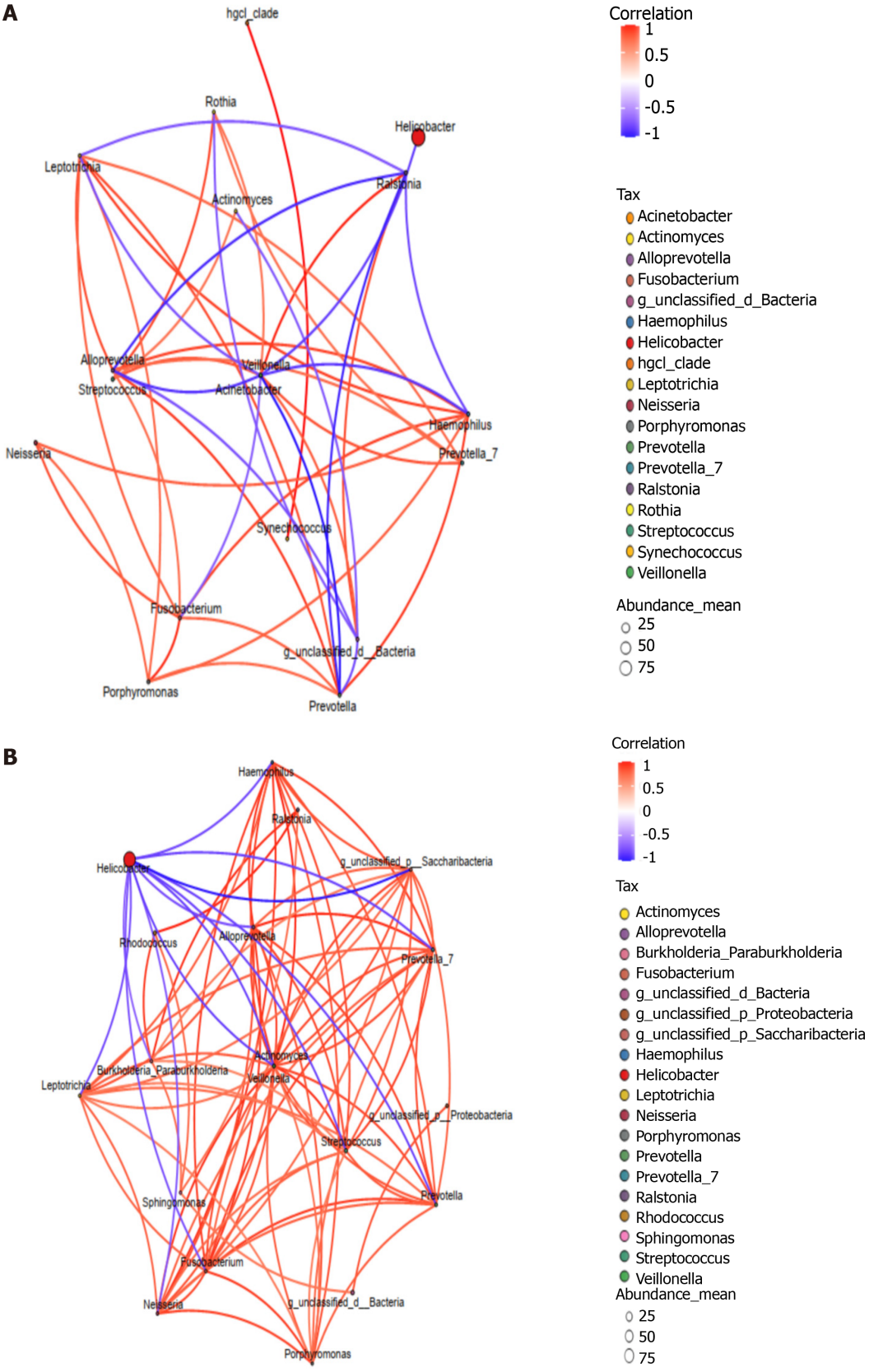


Figure 4 Single factor correlation network analysis including the 20 most abundant genera. A: Failure group; B: Success group. Each circle represents a species. Red and blue line represent positive and negative correlations. The thickness of the lines represents the correlation coefficient.

and 14-d triple therapy[24]. Previous studies have shown that *Lactobacillus* is a strong antagonist of *H. pylori* by preventing colonization and growth[25,26] and inhibiting adhesion to and invasion of gastric epithelial cells. *Lactobacillus* even has an antagonistic effect on multidrug resistant *H. pylori*[27]. In this study, the abundance of

Lactobacillus was significantly higher in the success group than in the failure group, confirming the beneficial effect of *Lactobacillus* in the eradication of *H. pylori*. Moreover, previous studies found changes in *Sphingomonas* in a variety of diseases[28,29]. In this study, it had a significant negative correlation with *H. pylori* and was more abundant in the success group than in the failure group.

Analysis of the microbiota against different gastric mucosa backgrounds showed that the genera *Veronococcus* and *Cilium* were enriched in chronic atrophic gastritis. Previous studies have shown bacterial overgrowth in the gastric mucosa of pre-gastric cancer. *Veronococcus* and *Cilium* are enriched in gastric mucosa of gastric cancer[30]. *Veronococcus* can convert nitrate to nitrite, and the increased concentration of nitrite may promote gastric cancer[31]. In addition, *Veronococcus* is enriched in patients with oral cancer, colorectal cancer, and lung cancer[32-35]. A study conducted in Colombia showed that the gastric mucosal flora of a population at high risk of gastric cancer was significantly enriched in *Veronococcus* and *Cilium* but the rate of *H. pylori* infection was not increased. Therefore, the genus *Veronococcus* and *Cilium* may be factors promoting the occurrence of gastric cancer. Our results confirmed a trend in pre-gastric cancer.

A negative correlation was found between *H. pylori* and other microbiota, which was more significant in the success group than in the failure group. The negative correlation of *H. pylori* and other microbiota was associated with a positive correlation among the other bacterial communities. Co-inhibition of *H. pylori* by a variety of mutually-promoting microbiota may improve the effectiveness of *H. pylori* eradication. There was no difference in the abundance of mutually-promoting microbiota present in the two groups. The lack of correlation between mutually-promoting microbiota and *H. pylori* in the failure group may have been related to a difference in *H. pylori* strains and needs to be confirmed by further studies.

Differences in the gastric microbiota may have contributed to *H. pylori* eradication failure, because all samples were collected before *H. pylori* eradication. This study has some limitations. It had a relatively small sample size because of the high eradication rate. The result preliminarily showed an effect of the gastric microbiota on *H. pylori* eradication, but studies that have larger sample sizes are needed to confirm these findings. This study did not determine *H. pylori* resistance, but the results provide clinically significant guidance for empirical treatment.

CONCLUSION

The effect of quadruple *H. pylori* eradication therapy containing bismuth depends on the gastric microbiota. A high rate of *H. pylori* eradication was associated with the presence of *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*. The study identified gastric microbiota beneficial to *H. pylori* eradication and laid a foundation for further research on how the gastric microbiota influences *H. pylori* eradication. In addition, the study results may help to improve the eradication rate of *H. pylori* in the future.

ARTICLE HIGHLIGHTS

Research background

There are complex interactions between *Helicobacter pylori* (*H. pylori*) and other microbial communities in the gastric microecological environment. Yet, it remains unclear whether the interactions affect the eradication of *H. pylori*.

Research motivation

The motivation was to explore the interaction between gastric microbiota and *H. pylori* and to determine the influence of gastric microbiota on the eradication of *H. pylori*.

Research objectives

To investigate the characteristics of the gastric mucosa microbiota with *H. pylori* infection and the influence on *H. pylori* eradication treatment. This may help improve the eradication rate of *H. pylori* in the future.

Research methods

Patients with *H. pylori* infection underwent gastroscopy and received treatment. Propensity matching analysis was conducted, including the number of patients who

did not respond to treatment. The gastric microbiota was assayed by high-throughput sequencing and subsequent analysis of alpha diversity, beta diversity, species correlations, and predicted metabolic pathways.

Research results

The main phyla in the two groups were the same in the eight failure group patients who did not respond well to therapy and the 16 success group patients and included Proteobacteria, Bacteroides, Firmicutes, Actinomycetes, and Fusobacteria. The high rate of *H. pylori* eradication was associated with *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*. *H. pylori* was negatively correlated with other bacterial genera, and more bacterial genera were directly related to *H. pylori* in the success group.

Research conclusions

The effectiveness of quadruple *H. pylori* eradication therapy containing bismuth depended on the gastric microbiota. The high rate of *H. pylori* eradication was associated with *Rhodococcus*, *Lactobacillus*, and *Sphingomonas*.

Research perspectives

This study laid a foundation for further research on the mechanism of the influence of the gastric microbiota on *H. pylori* eradication, which will help to improve the eradication rate of *H. pylori* in the future.

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META-ANALYSIS

Endoscopic submucosal dissection *vs* endoscopic mucosal resection for colorectal polyps: A meta-analysis and meta-regression with single arm analysis

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Abstract

BACKGROUND

Endoscopic submucosal dissection (ESD) has shown to be effective in management of colorectal neoplasm in the Asian countries, while its implementation in Western countries where endoscopic mucosal resection (EMR) is preferred is still debatable.

AIM

To compare the surgical, histological, and oncological outcomes between ESD and EMR in the treatment of colorectal polyps, with subgroup analysis comparing the efficacy of ESD and EMR between Japan and the rest of the world.

METHODS

Embase and Medline databases were searched from inception to October 2020 in accordance with PRISMA guidelines for studies comparing *en bloc*, complete resection, margin involvement, resection time, need for additional surgery, complications, and recurrence rate of ESD with EMR.

RESULTS

Of 281344 colorectal polyps from 21 studies were included. When compared to EMR, the pooled analysis revealed ESD was associated with higher *en bloc* and complete resection rate, and lower lateral margin involvement and recurrence.

was prepared and revised according to the PRISMA 2009 Checklist.

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ESD led to increased procedural time, need for additional surgery, and perforation risk. No significant difference in bleeding risk was found between the two groups. Meta-regression analysis suggested only right colonic polyps correlated with an increased perforation risk in ESD. Confounders including polyp size and invasion depth did not significantly influence the *en bloc* and complete resection rate, bleeding risk and recurrence. In subgroup analysis, Japan performed better than the rest of the world in both ESD and EMR with perforation risk of 4% and 0.0002%, respectively, as compared to perforation risk of 8% and 1%, respectively, in reports coming from rest of the world.

CONCLUSION
ESD resulted in better resection outcomes and lower recurrence compared to EMR. With appropriate training, ESD is preferred over EMR as the first-line therapy for resection of colorectal polyps, without restricting to lesions greater than 20 mm and those with high suspicion of submucosal invasion.

Key Words: Endoscopic mucosal resection; Endoscopy; Colonic polyps; Colorectal neoplasm; Colonoscopy

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Core Tip: The present study is the most extensive meta-analysis evaluating the surgical, histological, and oncological outcomes of endoscopic submucosal dissection (ESD) in comparison to endoscopic mucosal resection (EMR) in the treatment of colorectal polyps. Our analysis also showed the increased proficiency in performing ESD and EMR in Japan as compared to the rest of the world.

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INTRODUCTION

In recent years, the incidence of colorectal polyps has increased drastically with the widespread implementation of national colorectal cancer (CRC) screening programs. CRC screening with colonoscopy provides the opportunity to identify and remove any precursor lesions[1] and polypectomy has been shown to reduce the incidence and mortality of CRC[1-3]. Currently, 2 major forms of polypectomy are performed, namely endoscopic submucosal dissection (ESD) and endoscopic mucosal resection (EMR) with the latter procedure favored by western countries.

ESD was first proposed in 1999 as an endoscopic resection technique for safe *en bloc* removal of superficial lesions in the upper gastrointestinal tract[4]. Although colorectal ESD is widely practiced in the Asian countries, the implementation of ESD in Western countries where endoscopic mucosal resection is preferred is still debatable. According to the 2015 European Society of Gastrointestinal Endoscopy (ESGE), ESD is strongly recommended when there is high suspicion of submucosal invasion as determined by morphologic factors and advanced surface pattern, especially in lesions greater than 20 mm[5]. Despite the technical difficulties faced, ESD is designed for *en bloc* resection of lesions in an attempt to achieve complete resection, reliable histopathological analysis, and reduced recurrence rate.

Previous meta-analyses and systematic reviews comparing ESD with EMR mainly focused on data reported from Asian countries with limited data coming from outside of Japan, without controlling for confounders or lesions < 20 mm[6,7]. Since then, numerous studies comparing between ESD and EMR have been reported from the West and other countries[8-16]. We sought to perform an expanded meta-analysis to include lesions ≤ 20 mm, and incorporate regression analysis to control for

confounders.

MATERIALS AND METHODS

Search strategy and inclusion criteria

A search was conducted with reference to the PRISMA methodology on October 4, 2020 in Medline and Embase[17]. Keywords and Mesh terms were employed in the search strategy relating to the 'Colorectal', 'ESD' and 'EMR', the full list of terms included in the search can be found in the Supplementary Material 1. References were managed with Endnote X9. Two authors (XCL, KRYN) were involved in the sieving of the abstracts. Articles that compared between EMR and ESD for colorectal polyps were included in the search. Study designs included both retrospective and prospective cohorts, and case-controlled studies. Editorials, conferences abstracts, and commentaries were excluded in the review.

Data extraction

Based on the pre-determined inclusion criterion, article selection was performed by two authors with consensus from an independent third author should discrepancies arise. Information pertaining to the demographics (country, sample size, age, gender, polyp location, polyp size, polyp macroscopic type and depth of cancer invasion) and outcomes were extracted. Outcomes were limited to *en bloc* resection, complete resection, lateral and vertical margin involvement, lymphovascular invasion, mean operation time, additional surgery required, perforation, bleeding, and recurrence. *En bloc* resection was defined as the resection of the lesion in a single specimen, and complete resection was defined as the resection of the lesion with no marginal involvement of neoplastic tissue, as assessed by the pathologist. Additional surgical operations performed were considered in cases with intra-operative complications as a result of ESD or EMR or those with technical difficulties. Bleeding was considered both intra-operative and post-operative, and perforation was considered when diagnosed endoscopically during resection or radiologically by the presence of free air. Recurrence was defined as the detection of local or secondary primary tumors on interval colonoscopy. When estimates of mean and standard deviation (SD) were not available for continuous data, well established formulae were used to estimate mean and SD from median and range[18].

Statistical analysis and quality assessment

Three type of analysis were performed in this review. Firstly, a meta-analysis of proportions was used to pool results from the individual arms (ESD and EMR) using a freeman turkey double arsine transformation[19]. The freeman turkey double arsine transformation is recommended for single arm meta-analysis and necessary to stabilize the variance[20]. Next, comparative meta-analysis after a 0.5 continuity correction was to compare the difference in ESD and EMR, using risk ratios (RR) as the outcome of interest for dichotomous variables and weighted mean differences for continuous variables[21]. A 0.5 continuity correction was considered appropriate to incorporate 0 events into the meta-analysis. Regardless of heterogeneity measures (I^2, tau, Cochran Q test), all analysis was performed in random effects with the Dersimonian and Laird model[22,23]. Region (Japan *vs* rest of the world) was defined with refences to the manuscript country of origin. Publication bias was assessed with harbord test and egger regression for dichotomous and continuous variables respectively when > 10 studies were available. A univariate meta regression was also conducted with in random effects using the restricted maximum likelihood model with Kapp variance estimator to adjust for confounders in the outcomes[24]. A subgroup analysis was considered to compare between the articles originating from Japan and outside of Japan and a sensitivity analysis was performed to only include studies that have polyps size of ≥ 20 mm. All articles were graded with the Newcastle Ottawa scale for cohort studies, grading articles on the domains of study group selection, study group comparability, and ascertainment of outcomes of interest[25]. The statistical methods of this study were reviewed by Cheng Han Ng (ORCID ID: 0000-0002-8297-1569) from Yong Loo Lin School of Medicine, National University of Singapore.

RESULTS

Study selection

A systematic search of the literature using our search strategy yielded 1180 articles. After removal of duplicates, 895 were excluded based on study title and abstract, and 212 underwent full text review, of which 21 articles comparing ESD with EMR in resection of colorectal polyps were subsequently included in the meta-analysis (Figure 1)[8-16,26-37]. In total, 12 originated from Japan[26-37], five from European[10-13,16], three from South Korea[8,9,14] and one from China[15]. Of the 21 studies, there were 17 retrospective cohort studies, two prospective cohort studies and two retrospective case-control studies. In total, 281344 colorectal polyps were resected, of which 19573 underwent ESD while 261771 underwent EMR. Quality assessment of those included studies mostly scored 6-7. A summary of the study characteristics of the included articles can be found in the Supplementary Table 1.

Overall results

The summary of the results is found in Table 1. The pooled analysis showed a higher rate of *en bloc* resection (RR = 1.837; 95%CI: 1.464 to 2.305; $P < 0.001$), and a lower frequency of positive lateral margin involvement (RR = 0.292; 95%CI: 0.089 to 0.995; $P = 0.042$) in the ESD group than the EMR group. Publication bias was significant for *en bloc* resection rate ($P = 0.0025$). No significant difference in the rates of positive vertical margin involvement was observed between ESD and EMR groups (RR = 4.368; 95%CI: 0.409 to 46.710; $P = 0.223$). However, the rate of complete resection was higher in the ESD compared to EMR groups (RR = 1.504; 95%CI: 1.041 to 2.174; $P < 0.03$). Significantly, the time taken for ESD was longer than EMR (RR = 72.709; 95%CI: 54.487 to 90.931; $P < 0.001$). ESD group required more additional surgical operations relative to that of EMR group (RR = 3.139; 95%CI: 1.360 to 7.243; $P = 0.007$).

Compared to EMR, ESD shows a significant increased risk of perforation (RR = 7.597; 95%CI: 4.281 to 13.479; $P < 0.001$) (Figure 2), but no significant difference in the bleeding risk was observed between the two groups (RR = 1.277; 95%CI: 0.896 to 1.820; $P = 0.175$) (Figure 3). The rate of recurrence in the ESD group was lower than that of the EMR group (RR = 0.269; 95%CI: 0.112 to 0.648; $P = 0.003$) (Figure 4).

Meta regression

The influence of the included covariates on *en bloc* resection, complete resection, risk of perforation and bleeding, and rate of recurrence evaluated by meta-regression are summarized in Table 2. Meta-regression analysis for risk of perforation suggested that right colonic polyps ($\beta = 7.731$; 95%CI: 4.965 to 10.497; $P < 0.001$) correlated with an increased risk in perforation in ESD (Figure 5). Other factors including polyp size and depth of invasion, did not influence the *en bloc* resection rate, complete resection rate, the risk of bleeding, and recurrence rate.

Sensitivity analysis for articles ≥ 20 mm

The results of the sensitivity analysis are summarized in Supplementary Table 2 and results were largely unchanged after a sensitivity analysis. When a sensitivity analysis was performed to include ≥ 20 mm colorectal polyps only, the pooled analysis revealed higher rate of *en bloc* resection (RR = 1.932; 95%CI: 1.389 to 2.688; $P < 0.001$), longer operation time (RR = 3.247; 95%CI: 59.249 to 87.245; $P < 0.001$), higher risk of perforation (RR = 4.513; 95%CI: 2.531 to 8.046; $P < 0.001$) and lower recurrence rate (RR = 0.191; 95%CI: 0.085 to 0.431; $P < 0.001$) in ESD compared to EMR groups. Furthermore, in the two studies included, more additional surgical operations were required (RR = 3.139; 95%CI: 1.360 to 7.243; $P = 0.007$) in the ESD than in the EMR groups.

Subgroup analysis by region

The results of the subgroup analysis comparing outcomes of ESD with EMR in Japan *vs* the rest of the world are presented in Table 3.

ESD in Japan had an *en bloc* resection rate of 89% (95%CI: 0.77 to 0.97), perforation risk of 4% (95%CI: 0.01 to 0.07), bleeding risk of 2.4% (95%CI: 0.01 to 0.04) and recurrence rate of 1% (95%CI: 0.01 to 0.02) while its EMR had an *en bloc* resection rate of 53% (95%CI: 0.38 to 0.67), perforation risk of 0.0002% (95%CI: 0.00 to 0.00081), bleeding risk of 2.1% (95%CI: 0.01 to 0.03) and recurrence rate of 7% (95%CI: 0.02 to 0.15). Comparing between the two techniques in Japan, ESD had higher *en bloc* resection rate (RR = 1.658, 95%CI: 1.270 to 2.165, $P < 0.001$), perforation risk (RR = 9.586, 95%CI: 4.425 to 20.768, $P < 0.001$) and bleeding risk (RR = 1.267, 95%CI: 1.174 to

Table 1 Pooled proportions and comparative meta-analysis of endoscopic submucosal dissection and endoscopic mucosal resection

	Total papers	Sample size (ESD)	Pooled proportions	Sample size (EMR)	Pooled proportions	RR (CI)	*P* value	Publication bias
En bloc resection	11	1641	89% (0.83-0.94)	1411	47% (0.36-0.59)	1.837 (1.464-2.305)	< 0.001	0.0025
Positive lateral margin	2	123	3% (0.01-0.06)	187	14% (0.09-0.19)	0.292 (0.089-0.995)	0.042	-
Positive vertical margin	1	38	5% (0.00-0.17)	83	1% (0.00-0.07)	4.368 (0.409-46.710)	0.223	-
Complete resection	8	918	82% (0.74-0.88)	1012	56% (0.34-0.77)	1.504 (1.041-2.174)	0.03	-
Lymphovsacular invasion	1	54	6% (0.03-0.13)	23	0% (0.00-0.04)	4.352 (0.248-76.483)	0.315	-
Mean procedural time	8	1087	-	838	-	72.709 (54.487-90.931)	< 0.001	-
Additional surgery	2	99	13% (0.07-0.21)	153	5% (0.02-0.09)	3.139 (1.360-7.243)	0.007	-
Perforation	18	19470	5% (0.03-0.09)	260901	0% (0.00-0.01)	7.597 (4.281-13.479)	< 0.001	0.301
Bleeding	14	20048	3% (0.02-0.05)	257065	3% (0.02-0.04)	1.277 (0.896-1.820)	0.175	0.139
Recurrences	12	1822	2% (0.01-0.03)	37721	10% (0.04-0.17)	0.269 (0.112-0.648)	0.003	0.725

ESD: Endoscopic submucosal dissection; EMR: Endoscopic mucosal resection; CI: Confidence interval.

1.367, $P < 0.001$) than EMR. Following sensitivity analysis, similar results were obtained, except ESD had lower recurrence rate than EMR (RR = 0.204, 95%CI: 0.097 to 0.429, $P < 0.001$), and there was no difference in bleeding risk (RR = 0.895, 95%CI: 0.438 to 1.829, $P = 0.762$) between the two techniques.

With regards to studies from the rest of the world, ESD had an *en bloc* resection rate of 90% (95%CI: 0.85 to 0.94), perforation risk of 8% (95%CI: 0.05 to 0.12), bleeding risk of 5% (95%CI: 0.02 to 0.11) and recurrence rate of 3% (95%CI: 0.00 to 0.08) while EMR had an *en bloc* resection rate of 41% (95%CI: 0.22 to 0.61), perforation risk of 1% (95%CI: 0.00 to 0.02), bleeding risk of 3% (95%CI: 0.01 to 0.06) and recurrence rate of 16% (95%CI: 0.04 to 0.32). Comparing between the two techniques, ESD had higher *en bloc* resection rate (RR = 2.201, 95%CI: 1.411 to 3.433, $P = 0.001$), perforation rate (RR = 4.602, 95%CI: 2.729 to 7.759, $P < 0.001$) and lower recurrence rate (RR = 0.245, 95%CI: 0.073 to 0.819, $P = 0.022$) than EMR. Following sensitivity analysis, similar results were obtained.

DISCUSSION

The advancements in endoscopic resection techniques have resulted in the shift from radical surgery to minimally invasive and organ-sparing endoscopic resection techniques, such as ESD and EMR, for the treatment of colorectal lesions. With reference to 2015 ESGE guidelines, ESD should be considered for colorectal lesions larger than 20 mm, with high suspicion of submucosal invasion, or those where *en bloc* resection by EMR are not feasible[5]. Previous meta-analyses comparing ESD and EMR for colorectal polyps primarily reported data from Asian countries, with 72.7% of the published studies from Japan[6]. Since then, several retrospective and prospective studies comparing ESD and EMR for the treatment of colorectal polyps have been published outside of Japan. The present study is the most extensive meta-analysis evaluating the surgical, histological, and oncological outcomes of ESD in comparison to EMR in the treatment of colorectal polyps. Nine out of 21 studies (42.9%) on colorectal polyps included in this meta-analysis were conducted in countries outside of Japan. While ESD has been known to provide significantly better resection outcomes and lower recurrence rate, our analysis found that polyp size and depth of invasion did not significantly influence the *en bloc* and complete resection rate,

Table 2 Meta-regression

	En bloc resection			Complete resection			Perforation			Bleeding			Recurrence		
	n	Beta (CI)	*P* value	*n*	Beta (CI)	*P* value	*n*	Beta (CI)	*P* value	*n*	Beta (CI)	*P* value	*n*	Beta (CI)	*P* value
Age	8	-0.017 (-0.168-0.133)	0.785	5	-0.010 (-0.426-0.406)	0.944	9	0.073 (-0.191-0.336)	0.535	8	-0.201 (-0.540-0.138)	0.197	8	0.254 (-0.039-0.547)	0.078
Gender	6	-6.023 (-23.152-11.106)	0.384	4	-20.662 (-108.758-67.434)	0.419	8	-7.868 (-22.078–6.342)	0.224	7	-4.446 (-27.915-19.022)	0.647	6	-9.096 (-62.174-43.982)	0.659
Polyp size	10	0.242 (-0.037-0.086)	0.390	6	-0.034 (-0.098-0.030)	0.217	10	-0.034 (-0.117-0.049)	0.378	9	-0.041 (-0.177-0.095)	0.498	8	0.077 (-0.118-0.272)	0.369
Lateral spreading tumor	10	1.262 (-1.072-3.596)	0.248	7	-0.481 (-4.655-3.693)	0.779	11	-2.324 (-5.536-0.888)	0.136	9	-0.680 (-5.586-4.226)	0.753	9	3.827 (-2.323-9.977)	0.185
Right colon	8	1.247 (-2.024-4.517)	0.387	5	2.373 (-0.881-5.626)	0.103	8	7.731 (4.965-10.497)	< 0.001	7	1.688 (-12.442-15.817)	0.771	6	-8.739 (-26.616-9.139)	0.246
Colon	7	-1.803 (-4.486-0.880)	0.145	4	-1.914 (-7.213-3.385)	0.26	8	0.759 (-3.184-4.701)	0.654	6	6.649 (-5.182-18.480)	0.194	7	-1.102 (-4.754-2.551)	0.473
Rectum	7	1.803 (-0.880-4.486)	0.145	4	1.914 (-3.385-7.213)	0.26	8	-0.759 (-4.701-3.184)	0.654	6	-6.649 (-18.480-5.182)	0.194	7	1.102 (-2.551-4.754)	0.473
Submucosal invasion	8	-0.004 (-0.014-0.007)	0.431	5	-0.006 (-0.020-0.008)	0.253	9	0.001 (-0.049-0.051)	0.956	8	-0.016 (-0.033-0.001)	0.061	7	-6.508 (-27.976-14.960)	0.471
Muscularis propria invasion	8	-5.870 (-17.738-5.998)	0.272	5	-0.157 (-21.715-21.401)	0.983	9	6.731 (-33.772-47.234)	0.706	8	-109.836 (-1040.943-821.272)	0.783	-	-	-

CI: Confidence interval.

bleeding and perforation risk, and recurrence rate in colorectal polyps that was not previously reported. Additionally, previous reviews were confined only to sessile lesions larger than 20 mm[6,7]. Our analysis also showed the increased proficiency in performing ESD and EMR in Japan as compared to the rest of the world.

Consistent with previous studies, ESD showed benefits in the technical, histological, and oncological outcomes. Pooled analysis showed higher rates of *en bloc* resection and complete resection in ESD than in EMR albeit significant publication bias ($P = 0.0025$). *En bloc* resection offers the technical advantage of removing the entire pathologic specimen, thus allowing for detailed histologic evaluation. This results in an increase in the complete resection rate which in turn reduces the recurrence rate. Therefore, *en bloc* resection with ESD is favored as it provides curative treatment without the need for surgery for lesions with significant likelihood of submucosal invasion[38]. However, the advantages of ESD come at the expense of longer procedural time, additional surgical operations, and increased perforation risk compared to EMR[6]. The high rate of additional surgical operations for ESD is presumed to be due to the aggressive selection of ESD for T1 Lesions. Although the perforation risk was higher with ESD, most perforations in the studies included were treated conservatively or endoscopically using endoclips[9,12,31,33,34,37].

Meta-regression was performed to assess the risk factors that affect surgical, histological, and oncological outcomes. Our present analysis showed that polyp size did not affect the risk of perforation, which was reported otherwise in studies by Kim

Table 3 Subgroup analysis (Japan and rest of the world) of pooled proportions and comparative meta-analysis of endoscopic submucosal dissection and endoscopic mucosal resection

	Japan papers	Pooled proportions (ESD)	Pooled proportions (EMR)	RR (CI)	*P* value	Rest of the world papers	Pooled proportions (ESD)	Pooled proportions (EMR)	RR (CI)	*P* value
Overall results										
En bloc resection	6	89% (0.77-0.97)	53% (0.38-0.67)	1.658 (1.270-2.165)	< 0.001	5	90% (0.85-0.94)	41% (0.22-0.61)	2.201 (1.411-3.433)	0.001
Positive lateral margin	2	3% (0.00-0.06)	14% (0.09-0.19)	0.292 (0.089-0.955)	0.042	-	-	-	-	-
Positive vertical margin	1	5% (0.01-0.17)	1% (0.00-0.05)	4.368 (0.409-46.710)	0.223	-	-	-	-	-
Complete resection	2	79% (0.73-0.84)	53% (0.48-0.58)	1.452 (1.303-1.618)	< 0.001	6	85% (0.78-0.91)	59% (0.27-0.88)	1.562 (0.921-2.650)	0.098
Lymphovsacular invasion	1	6% (0.03-0.13)	0% (0.00-0.04)	4.352 (0.248-76.483)	0.315	-	-	-	-	-
Mean procedural time	4	-	-	72.106 (48.831-95.382)	< 0.001	4	-	-	73.916 (36.075-111.757)	< 0.001
Additional surgery	2	13% (0.07-0.21)	5% (0.02-0.09)	3.139 (1.360-7.243)	0.007	-	-	-	-	-
Perforation	11	4% (0.01-0.07)	0.0002% (0.00-0.00081)	9.586 (4.425-20.768)	< 0.001	7	8% (0.05-0.12)	1% (0.00-0.02)	4.602 (2.729-7.759)	< 0.001
Bleeding	8	2.4% (0.01-0.04)	2.1% (0.01-0.03)	1.267 (1.174-1.367)	< 0.001	6	5% (0.02- 0.11)	3% (0.01-0.06)	1.986 (0.716-5.508)	0.188
Recurrences	7	1% (0.01-0.02)	7% (0.02-0.15)	0.274 (0.071-1.054)	0.06	5	3% (0.00-0.08)	16% (0.04-0.32)	0.245 (0.073-0.819)	0.022
Sensitivity analysis (≥ 20 mm only)										
En bloc resection	4	82% (0.72-0.91)	50% (0.33-0.67)	1.645 (1.174-2.306)	0.004	2	91% (0.88-0.93)	33% (0.28-0.37)	2.668 (1.752-4.063)	< 0.001
Positive lateral margin	2	3% (0.00-0.06)	14% (0.09-0.19)	0.292 (0.089-0.955)	0.042	-	-	-	-	-
Positive vertical margin	1	5% (0.01-0.17)	1% (0.00-0.07)	4.368 (0.409-46.710)	0.223	-	-	-	-	-
Complete resection	2	79% (0.73-0.84)	53% (0.48-0.58)	1.452 (1.303-1.618)	< 0.001	2	90% (0.87-0.93)	77% (0.73-0.82)	1.613 (0.209-12.452)	0.647
Lymphovsacular invasion	2	6% (0.03-0.13)	0% (0.00-0.04)	4.352 (0.248-76.483)	0.315	-	-	-	-	-
Mean procedural time	2	-	-	69.167 (48.446-89.888)	< 0.001	1	-	-	82.700 (67.578-97.822)	< 0.001
Additional surgery	2	13% (0.07-0.21)	5% (0.02-0.09)	3.139 (1.360-7.243)	0.007	-	-	-	-	-
Perforation	5	4% (0.01-0.07)	0% (0.00-0.01)	5.235 (2.123-12.910)	< 0.001	3	7% (0.05-0.10)	1% (0.00-0.03)	4.546 (1.674-12.346)	0.003

Bleeding	4	3% (0.00-0.07)	3% (0.01-0.05)	0.895 (0.438-1.829)	0.762	3	3% (0.00-0.08)	1% (0.00-0.04)	2.233 (0.489-10.197)	0.300
Recurrences	5	1% (0.00-0.02)	7% (0.02-0.15)	0.204 (0.097-0.429)	< 0.001	3	3% (0.00-0.10)	27% (0.10-0.20)	0.179 (0.032-0.990)	0.049

ESD: Endoscopic submucosal dissection; EMR: Endoscopic mucosal resection; RR: Relative risk; CI: Confidence interval.

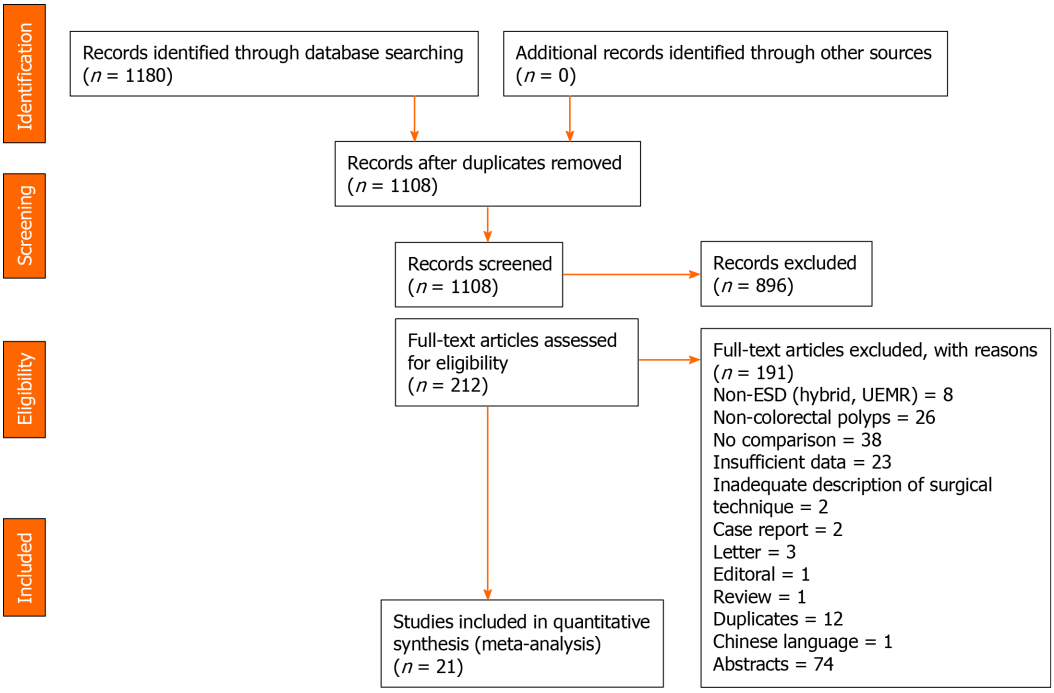


Figure 1 PRISMA flow diagram of included articles. ESD: Endoscopic submucosal dissection; EMR: Endoscopic mucosal resection.

et al[39] and Hong *et al*[40] Polyp size and depth of invasion were also not associated with significant change in *en bloc* and complete resection rate, risk of bleeding, and recurrence between ESD and EMR. Furthermore, the *en bloc* resection rate (before RR = 1.837, 95%CI: 1.464 to 2.305, $P < 0.001$; after RR = 1.932, 95%CI: 1.389 to 2.688, $P < 0.001$) and recurrence rate (before RR = 0.269, 95%CI: 0.112 to 0.648, $P = 0.003$; after RR = 0.191, 95%CI: 0.085 to 0.431; $P < 0.001$) appeared comparable before and after the sensitivity analysis to ≥ 20 mm polyps. Instead, the risk of perforation was increased in patients with right colonic polyps and this was consistent with previous study which identified the technical difficulty and proficient endoscopic skills required to remove polyps from right colon safely[41]. As such, training should ensure endoscopists achieve procedural proficiency in left sided lesions before proceeding to attempt right sided lesions. Other factors including polyp size and depth of invasion are less important criteria when deciding between EMR and ESD in skilled tertiary centers.

Despite the advantages of ESD, the data regarding the efficacy of colorectal ESD have been inconsistent and vary between Japan and the rest of the world. One of the reasons is the limitations to the implementation of ESD in other countries, which are in part due to the lack of expertise and training centers. To date, no meta-analysis comparing ESD and EMR between Japan and the rest of the world have been performed. Our single arm meta-analysis found that Japan performed better than the rest of the world in ESD and EMR. Significantly, perforation is a major concern in ESD. The perforation risk of ESD and EMR was 4% and 0.0002%, respectively in Japan, and 8% and 1%, respectively in the rest of the world. While there is an observed increase risk of perforation from Japan only studies (RR = 9.586) compared to the rest of the world (RR = 4.602), even after sensitivity analysis, the risk of perforation for ESD was only statistically higher in Japan only studies due to the very low risk of perforation in EMR in Japan. In addition, the challenges of doing ESD in difficult could have resulted in the higher perforation rate in ESD compared with EMR in Japan. Another important factor to consider is the recurrence rate of ESD and EMR which was 1% and 7%,

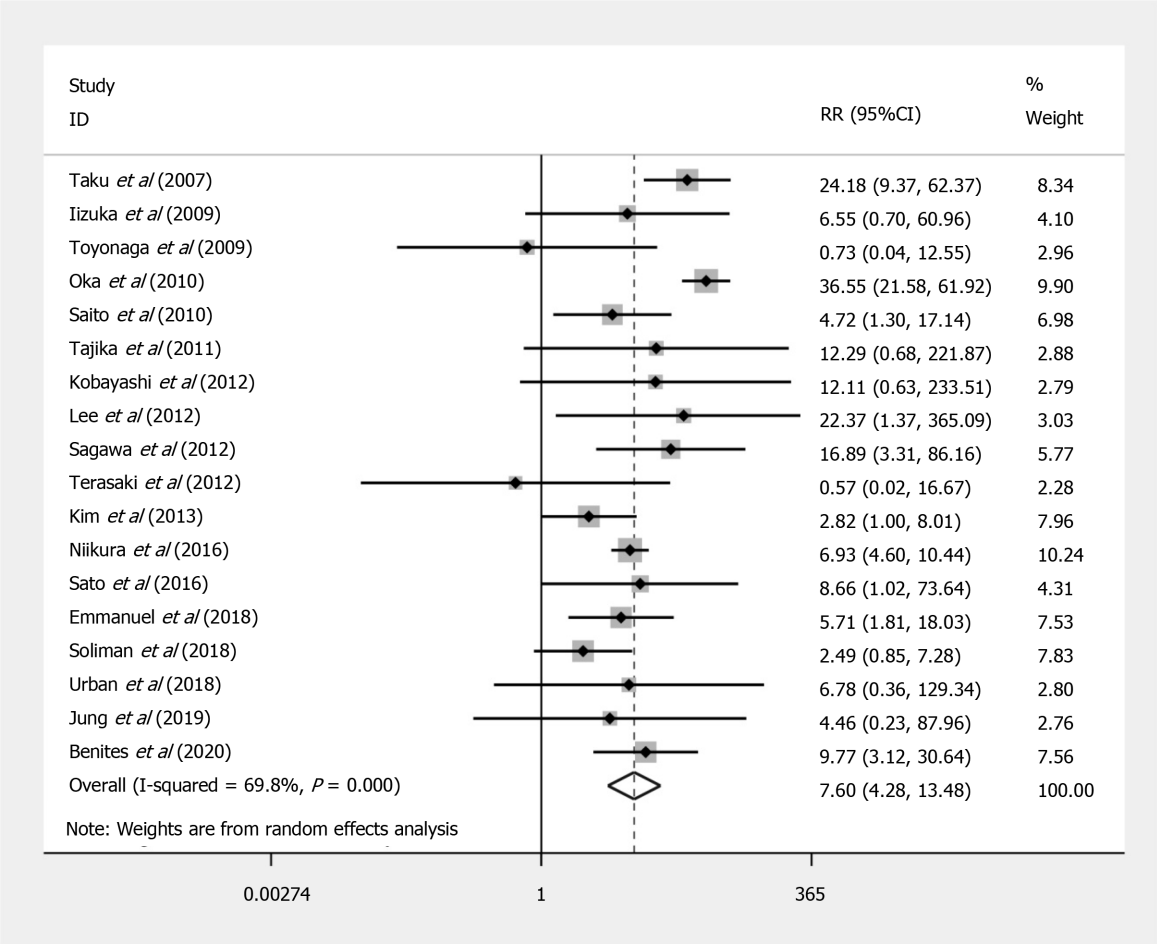


Figure 2 Forest plot for risk of perforation. RR: Relative risk; CI: Confidence interval.

respectively in Japan and 3% and 27%, respectively in the rest of the world following sensitivity analysis. The observed increase in recurrence rate from Japan only studies (RR = 0.204) compared to the rest of the world (RR = 0.179) was due to the much lower recurrence rate of EMR in Japan as compared to the rest of the world. Overall, our results seem to favor studies from Japan and are in tandem with a single arm analysis of ESD only procedures with subgroup on region efficacy[42]. However, most of the studies originating from the rest of the world should be interpreted with the understanding that these studies are mainly from tertiary centers and the results may not be generalized to non-tertiary centers.

The potential of ESD resection is limited by the difficulty in conducting the procedure as the length of procedure for ESD even when performed by experienced endoscopists can be three times longer than that for EMR[43]. However, the advancement in endoscopic resection equipment has been shown to shorten the procedure time despite a relatively short training duration[44]. Using the cumulative sum method, Miyakawa *et al*[44] recently reported the use of Stag-Beetle Knife Jr for ESD in a Japan single-center study generated good learning curve to achieve satisfactory resection speed (min/cm^2), which allowed the acquisition of proficient and safe skills within 120 cases[44]. Other alternatives to ESD do exist, such as hybrid ESD and pre-cut EMR. This hybrid approach has been shown to have lower *en bloc* resection rate (68.4% *vs* 91.0%) and complete resection rate (60.6% *vs* 82.9%) than conventional ESD[42]. Currently, underwater EMR has been thought to be a safe and effective method with higher rate of *en bloc* resection and lower rate of recurrence[45], but no head-to-head comparisons have been done between UMER and ESD.

The inclusion of 21 studies with a total of 281344 polyps based on our search strategy and inclusion criteria represents the most extensive meta-analysis on this issue. However, as no randomized controlled trials comparing the performance between EMR and ESD have yet been conducted, this highlights the need for a randomized study to better understand the efficacy and safety of these techniques in the management of colorectal polyps. The evaluation of heterogeneity allowed us to compare the significant differences in the performance of ESD and EMR between

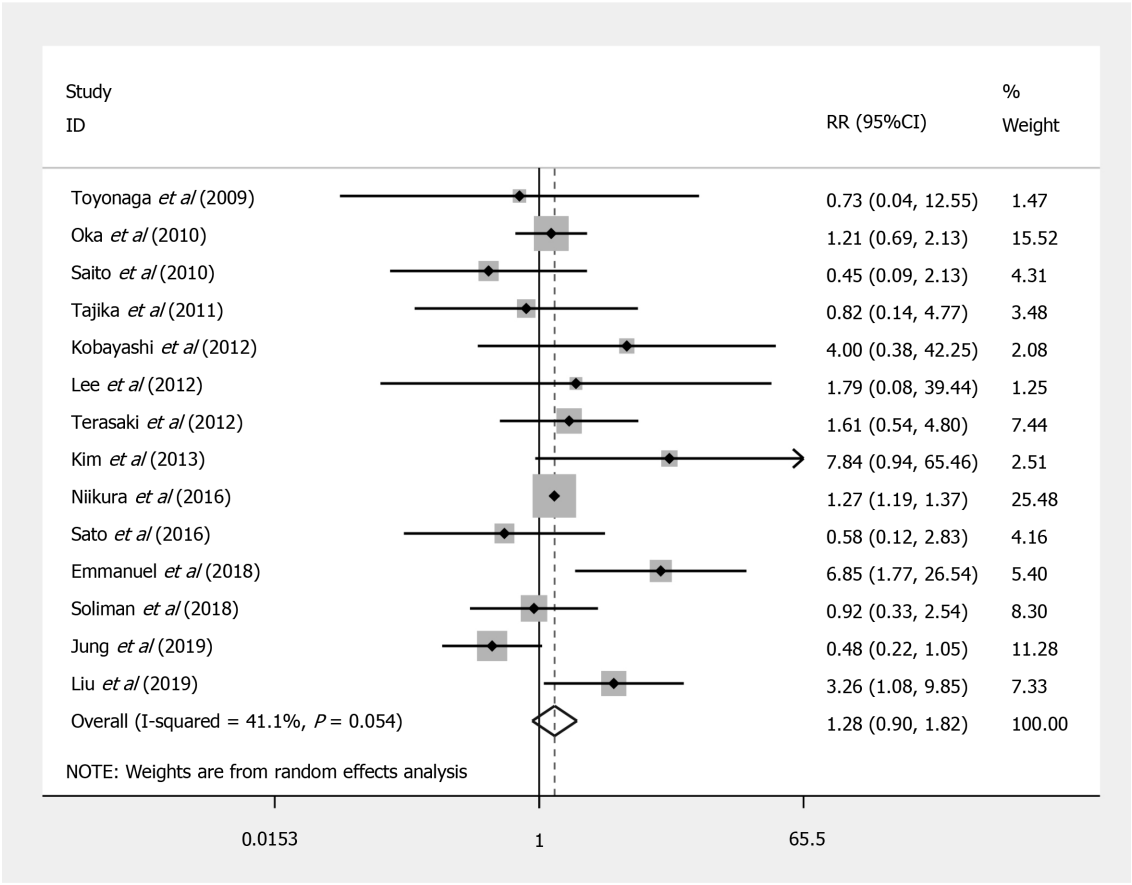


Figure 3 Forest plot for risk of bleeding. RR: Relative risk; CI: Confidence interval.

Japan and the rest of the world.

Our meta-analysis has some limitations. While we aimed to decrease heterogeneity, the included articles used a variety of EMR techniques including standard EMR, piecemeal EMR, EMR with small incision, EMR-precutting and EMR-circumferential incision. This, however, was an acceptable confounding factor in previous meta-analysis analysis. Also, a major factor that we were unable to regress for was the procedural skills of each centers. ESD and EMR are largely operator dependent and we were only able to account for it in a subgroup analysis comparing between studies conducted in Japan and the rest of the world.

CONCLUSION

Evidence from this meta-analysis suggests that with appropriate training, ESD is preferred over EMR as the first-line therapy for resection of colorectal polyps, without restricting to lesions greater than 20 mm and those with high suspicion of submucosal invasion. Our overall findings are consistent with previous meta-analyses showing ESD is associated with higher rate of *en bloc* and complete resection, and lower recurrence compared to EMR, but at the cost of increased procedural time, need for additional surgical operations and perforation risk. This is coupled with the new finding that confounders including polyp size and invasion depth did not influence the rates of *en bloc* and complete resection, bleeding risk, and recurrence.

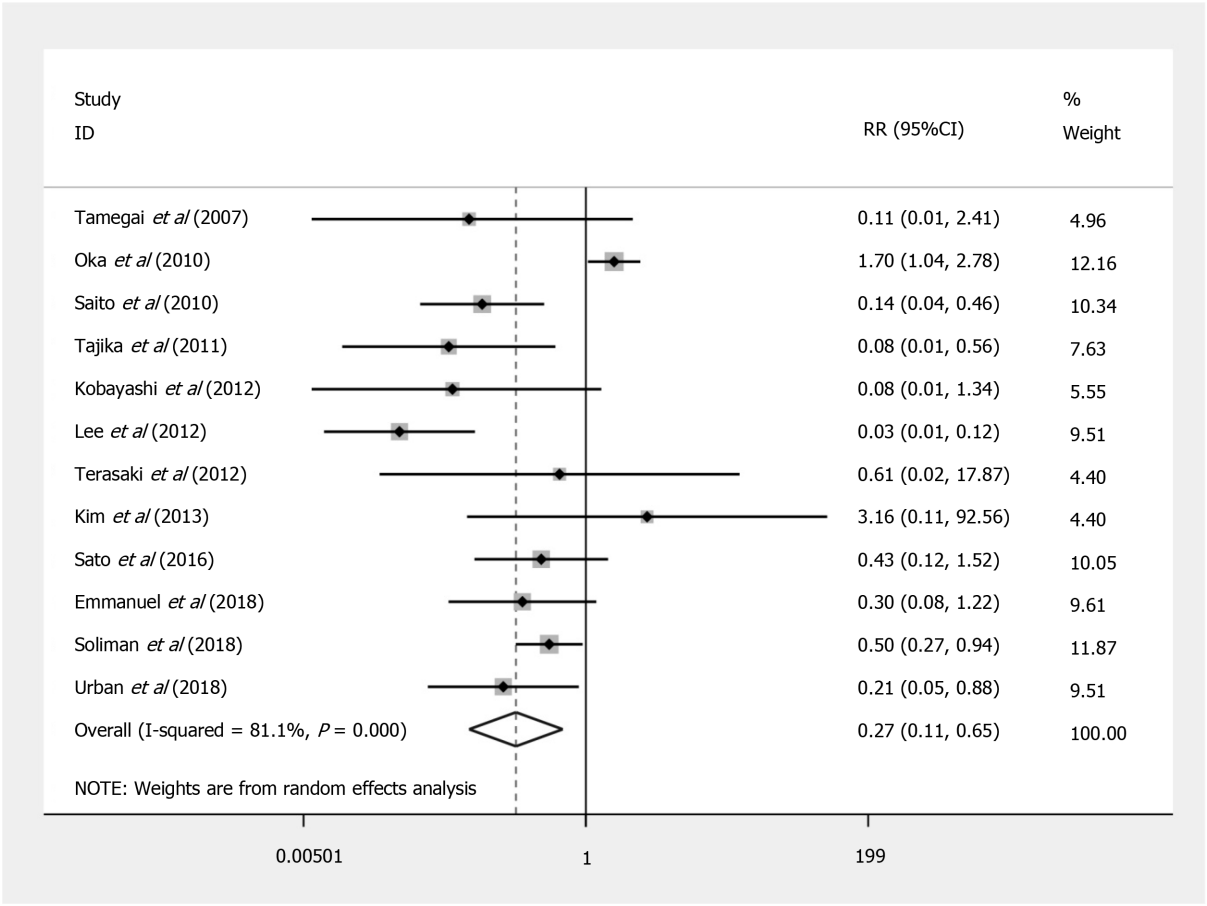


Figure 4 Forest plot for recurrence rate. RR: Relative risk; CI: Confidence interval.

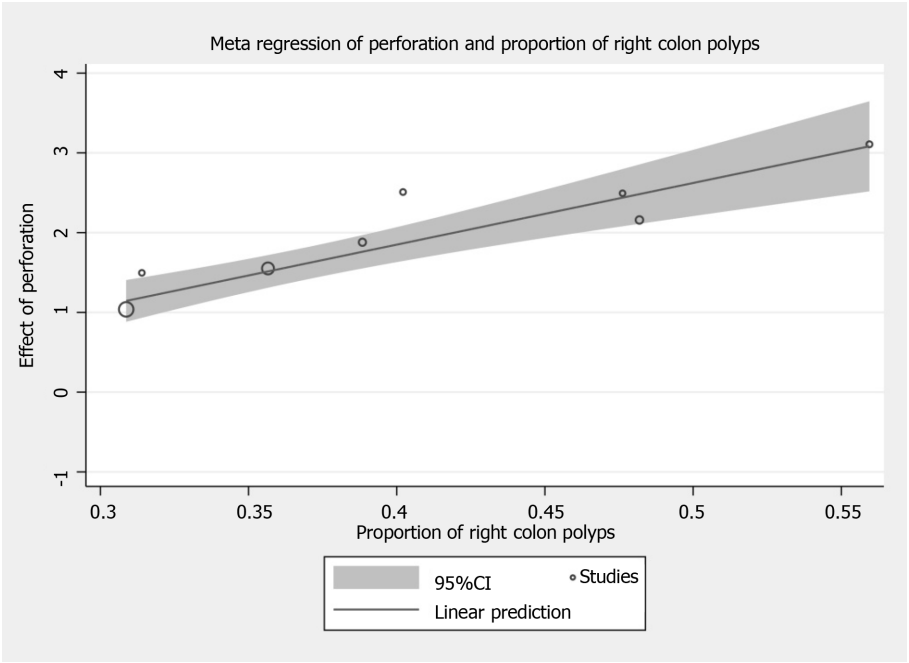


Figure 5 Meta regression of perforation and proportion of right colon polyps. CI: Confidence interval.

ARTICLE HIGHLIGHTS

Research background

Endoscopic submucosal dissection (ESD) has shown to be effective in management of colorectal neoplasm in the Asian countries, while its implementation in Western countries where endoscopic mucosal resection (EMR) is preferred is still debatable.

Research motivation

Previous meta-analyses and systematic reviews comparing ESD with EMR included studies mainly conducted in Asia, with limited data coming from outside of Japan and did not control for confounders or lesions smaller than 20 mm.

Research objectives

To compare the outcomes of ESD and EMR in the treatment of colorectal polyps, with subgroup analysis comparing the efficacy of these two techniques between Japan and the rest of the world.

Research methods

Embase and Medline databases were searched in accordance with PRISMA guidelines for studies comparing *en bloc*, complete resection, margin involvement, resection time, need for additional surgery, complications, and recurrence rate of ESD with EMR in patients with colorectal lesions.

Research results

ESD was associated with better resection outcomes and lower recurrence rate when compared to EMR. Meta-regression analysis suggested only right colonic polyps correlated with an increased perforation risk in ESD, while confounders including polyp size and invasion depth did not significantly influence the resection outcomes, bleeding risk and recurrence. Subgroup analysis showed that Japan performed better than the rest of the world in both ESD and EMR with lower perforation risk.

Research conclusions

This meta-analysis suggests that with appropriate training, ESD is preferred over EMR as the first-line therapy for resection of colorectal polyps, without restricting to lesions greater than 20 mm and those with high suspicion of submucosal invasion. Increased proficiency in performing ESD and EMR was shown in Japan as compared with the rest of the world.

Research perspectives

This highlights the need to establish adequate training programs for colorectal ESD to be performed effectively. A randomized controlled trial is necessary to better understand the efficacy and safety of these techniques in the management of colorectal polyps.

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CASE REPORT

Gastric schwannoma treated by endoscopic full-thickness resection and endoscopic purse-string suture: A case report

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Author contributions: Lu ZY reviewed the literature and was responsible for manuscript drafting and organization of illustrations and contributed to the interpretation of the imaging findings and endoscopic findings; Zhao DY analyzed and interpreted the pathological findings, immunohistochemical findings, and genetic mutation and was responsible for the revision of the manuscript for important intellectual content; All authors issued final approval for the version to be submitted.

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Abstract

BACKGROUND

Schwannomas, also known as neurinomas, are tumors that derive from Schwann cells. Gastrointestinal schwannomas are extremely rare, but the stomach is the most common site. Gastric schwannomas are usually asymptomatic. Endoscopy and imaging modalities might offer useful preliminary diagnostic information. However, to diagnose schwannoma, the immunohistochemical positivity for S-100 protein is essential, whereas CD117, CD34, SMA, desmin, and DOG-1 are negative.

CASE SUMMARY

A 45-year-old female was found to have a gastric mass during a medical examination, which was diagnosed as a gastric schwannoma. We performed endoscopic full-thickness resection and endoscopic purse-string suture. Pathology and immunohistochemical staining confirmed the diagnosis of gastric schwannoma through the positivity of S-100 protein. Furthermore, to exclude the misdiagnosis of gastrointestinal stromal tumor, we performed a mutational detection of the *c-Kit* and *PDGFRA* genes. Postoperative follow-up revealed that the patient recovered well.

CONCLUSION

Immunohistochemical staining is essential for the diagnosis of schwannoma. Endoscopic full-thickness resection is an effective treatment method for gastric schwannoma.

Key Words: Gastric schwannoma; Endoscopic full-thickness resection; Endoscopic purse-string suture; Immunohistochemical staining; Gene mutational analysis; Case report

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Core Tip: Schwannomas can occur in any part of the digestive tract but are most common in the stomach. Gastric schwannomas are typically asymptomatic, and it is difficult to make a precise preoperative diagnosis. The final diagnosis of schwannoma is based on immunohistochemical staining. We performed endoscopic full-thickness resection and endoscopic purse-string suture. We report a case diagnosed with gastric schwannoma.

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INTRODUCTION

Schwannomas, which are also known as neurinomas, were first described in 1910 by Verocay and are rarely observed in the gastrointestinal tract[1,2]. The stomach is the site with the highest incidence of schwannomas in the gastrointestinal tract[3]. Gastric schwannomas are typically asymptomatic, and the most common symptoms are stomachache, abdominal mass, and gastrointestinal hemorrhage[4,5].

Although endoscopy and imaging modalities, such as computed tomography (CT), magnetic resonance imaging, positron emission tomography-CT, might offer useful preliminary diagnostic information, it is still difficult to achieve the precise preoperative diagnosis of gastric schwannomas. Immunohistochemical positivity of S-100 protein is essential for the final diagnosis of schwannoma, whereas CD117, CD34, SMA, desmin, and DOG-1 are negative[6,7]. Gastric schwannomas are almost always benign with no recurrence or metastasis[6,8], and the optimal treatment for gastric schwannoma is surgical resection.

In our case, the schwannoma was discovered incidentally by abdominal CT. Gastroscopy and endoscopic ultrasonography (EUS) were then performed. Endoscopic full-thickness resection and endoscopic purse-string suture were performed. Finally, the diagnosis of gastric schwannoma was confirmed by histological, immunohistochemical, and gene mutational investigations.

CASE PRESENTATION

Chief complaints

A 45-year-old female had a gastric mass during a medical examination.

History of present illness

A 45-year-old female visited a local hospital for a regular health examination without any symptoms. The patient had an abdominal CT scan, which revealed a rounded mass arising from the greater curvature of the gastric body, suggesting a gastrointestinal stromal tumor (GIST) as a likely diagnosis. For further diagnosis and treatment, she was admitted to the Department of Gastroenterology at our hospital.

History of past illness

There was no other significant medical history. The patient had no history of prior gastroenterological symptoms. There was no relevant history including past interventions and outcomes.

Personal and family history

The patient had no history of smoking or drinking alcohol. Her occupation was a housewife. There was no relevant family history.

Physical examination

All vital signs of the patient were stable, and physical examination revealed no

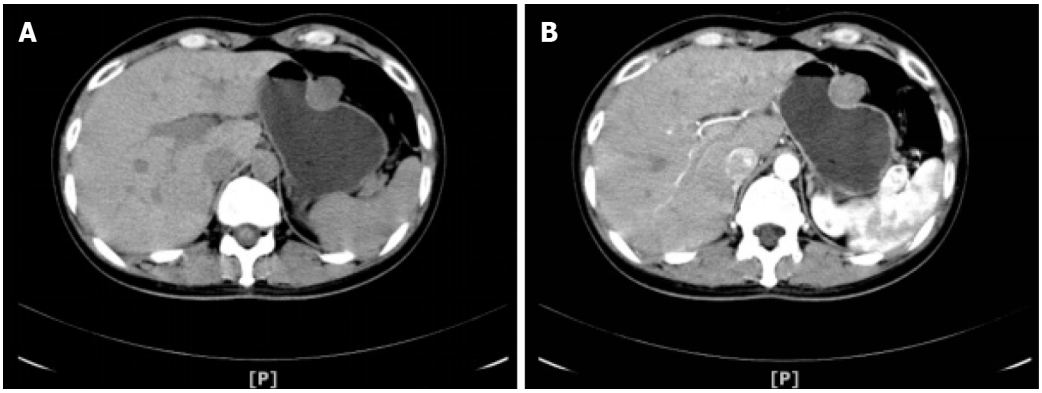


Figure 1 Abdominal computed tomography scanning. A: Computed tomography scan revealed a rounded mass arising from the greater curvature of the gastric body; B: The gastric mass exhibited slight internal contrast enhancement.

noteworthy positive sign.

Laboratory examinations

The levels of the tumor markers AFP, CA125, and CEA were in the normal range. Blood tests, fecal examinations, and coagulation function all demonstrated normal results.

Imaging examinations

Nine days before admission, the patient underwent abdominal CT scanning for a medical examination, and it revealed a 24.9 mm × 23.9 mm rounded mass arising from the greater curvature of the gastric body with slight internal contrast enhancement (Figure 1), suggesting a GIST as a likely diagnosis. No enlarged pericolic lymph nodes were observed.

Endoscopic examinations

Gastroscopy demonstrated a 2.0 cm × 1.8 cm hemispherical protrusion lesion of the gastric body, and EUS revealed hypoechoic and homogeneous echo lesions originating from the muscularis propria (Figure 2).

Pathological findings and immunohistochemical staining

Pathological analysis and immunohistochemical staining (Figure 3) confirmed the diagnosis of gastric schwannoma through positivity for S-100 protein, whereas CD117, CD34, α-SMA, desmin, and DOG-1 were negative.

Gene mutational analysis

To provide evidence for the differential diagnosis of GIST, we performed a mutational detection of the *c-Kit* and *PDGFRA* genes (Figure 4), and the results showed that no mutations were detected in the sample.

FINAL DIAGNOSIS

The final diagnosis of the presented case was gastric schwannoma.

TREATMENT

Endoscopic full-thickness resection and endoscopic purse-string suture were performed (Figure 5). Postoperatively, acid suppression, hemostasis, protection of the gastric mucosa, and nutritional support were administered.

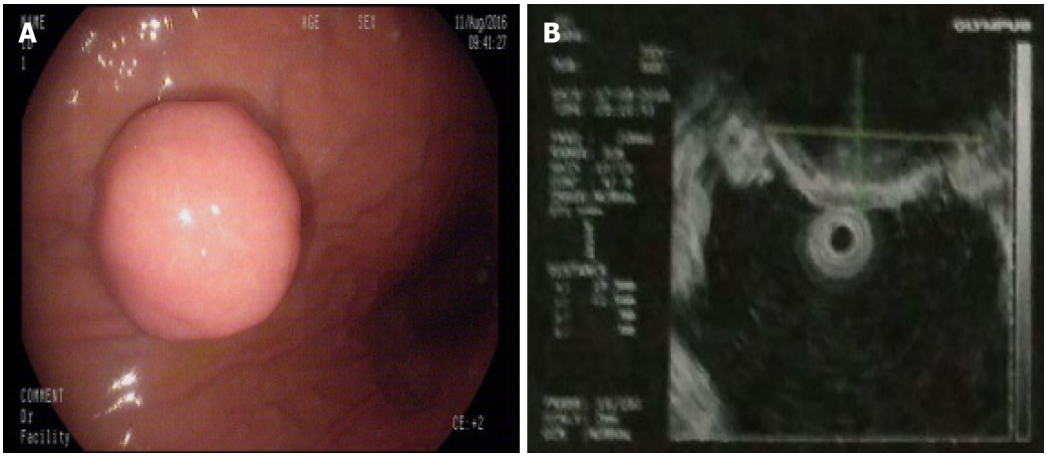


Figure 2 Gastroscopy and endoscopic ultrasonography. A: Gastroscopy demonstrated a hemispherical protrusion lesion of the gastric body; B: Endoscopic ultrasonography showed that the lesion arose from the muscularis propria.

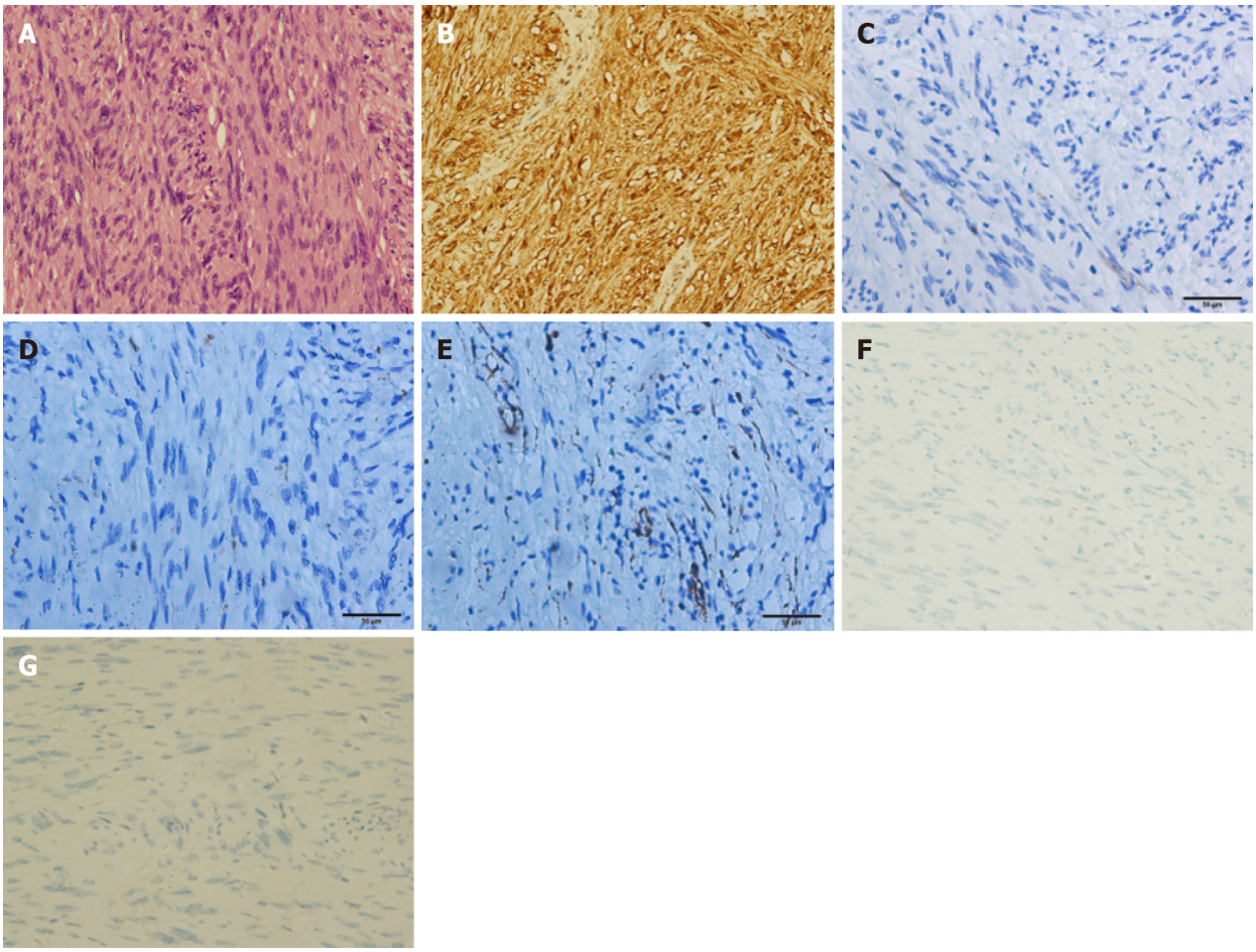


Figure 3 Pathological analysis and immunohistochemical staining. A: Hematoxylin and eosin staining revealed spindle cell tumors with mild cells, mitotic figures 1-2/50 high-power field, local inflammatory cell infiltration, and no necrosis. Combined with immunohistochemistry and gene detection results, the results were consistent with schwannoma; B-G: Immunohistochemical staining of the gastric mass confirmed a gastric schwannoma through positive staining for S-100 protein (B), whereas CD117 (C), CD34 (D), α-smooth muscle actin (E), desmin (F), and DOG1 (G) were negative.

OUTCOME AND FOLLOW-UP

The patient was well recovered and was discharged on her seventh day post operation. The patient was followed up for 16 mo after the operation. Gastroscopy was performed (Figure 6), and the results indicated that the incision recovered well.

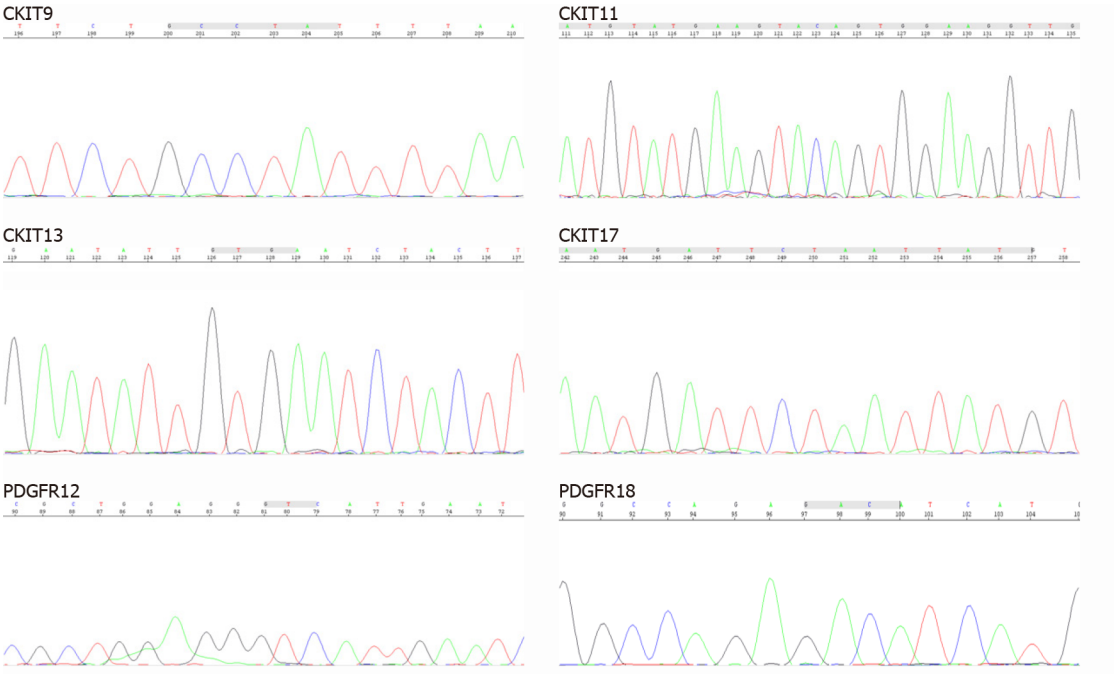


Figure 4 *c-Kit* and *PDGFRA* gene mutational analysis. DNA sequencing electropherograms revealed an absence of mutations in exons 9, 11, 13, and 17 of the *c-Kit* gene and exons 12 and 18 of the *PDGFRA* gene.

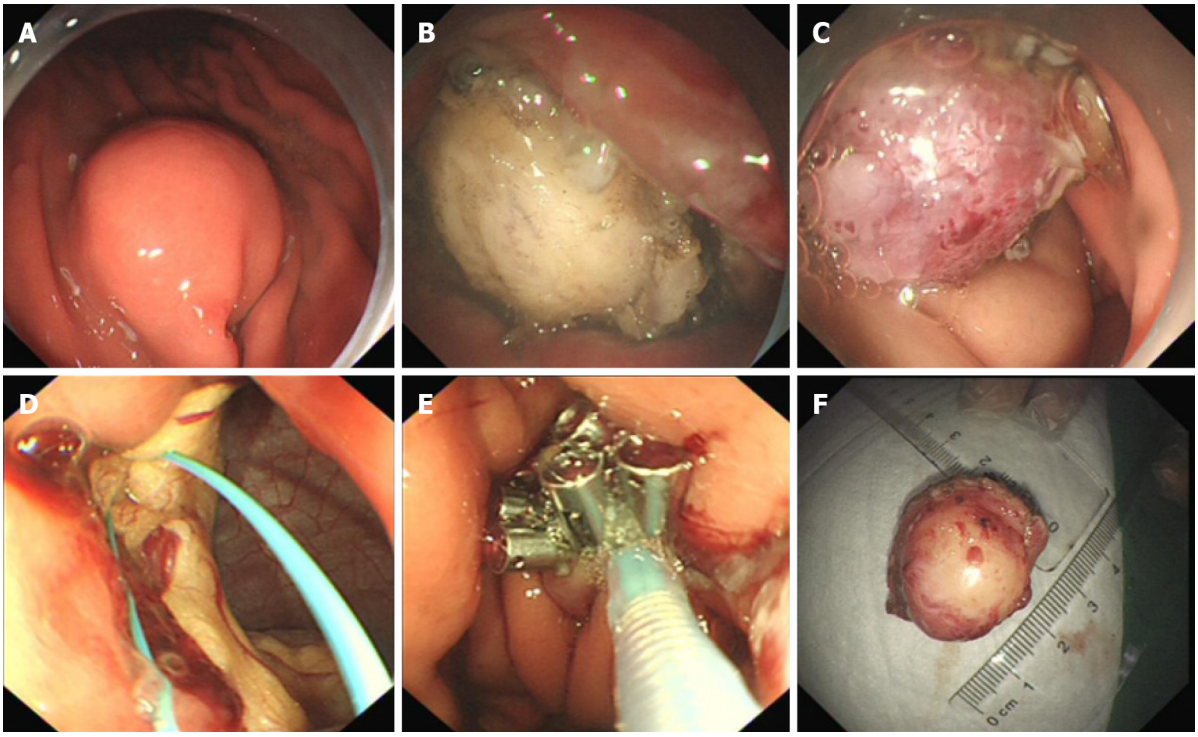


Figure 5 Endoscopic full-thickness resection operative process. A: Marked the lesion with argon plasma coagulation; B and C: Application of the insulated-tip knife to isolate the stromal tumor along its periphery; D and E: An "artificial perforation" observed after stromal tumor resection and sealed the perforation by endoscopic purse-string suture; F: The resected tumor.

DISCUSSION

Schwannomas are tumors originating from Schwann cells that usually affect the subcutaneous tissue of the distal limbs[9]. Schwannomas of the gastrointestinal tract represent approximately 3% of all mesenchymal tumors of the gastrointestinal tract [10]. In the gastrointestinal tract, the stomach is the site with the highest incidence of schwannomas followed by the colon[11]. The small intestine and esophagus are the

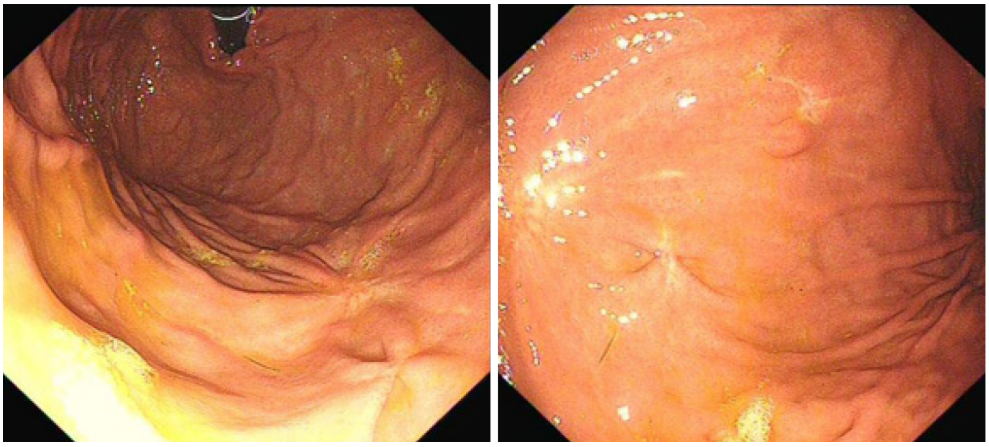


Figure 6 Gastroscopy at 16 mo after the operation revealed that the incision recovered well and that there was no recurrence.

most infrequently affected sites[12,13]. Gastric schwannomas account for 0.2% of all gastric tumors[9].

Diagnostic methods for gastric schwannomas, such as endoscopy, EUS, CT, magnetic resonance imaging, and positron emission tomography, have recently been proposed. On endoscopy, gastric schwannomas appear as elevated submucosal masses, with or without a central ulcer[14]. Endoscopic biopsy is not as effective as expected, as it can lead to false negative results[9]. On EUS evaluation, a rounded submucosal mass, a well-defined margin, heterogeneous hypoechogenicity or isoechogenicity, and deficiency of cystic change and calcification are significant for the diagnosis of gastric schwannoma[15-17].

Previous studies demonstrated that gastric schwannomas showed well-demarcated masses that are heterogeneous or homogeneous contrast enhancement on CT[14,18]. Ji *et al*[19] and Wang *et al*[20] reported that homogeneous progressive enhancement on dynamic CT was a characteristic finding of gastric schwannoma. On magnetic resonance imaging examination, the signal intensity of most gastric schwannomas is low to medium on T1-weighted images and high on T2-weighted images[21]. Recently, several cases of gastric schwannoma that were found with an increased uptake of fluorodeoxyglucose on positron emission tomography were reported[22]. Even with the above modern imaging modalities, it is still difficult to achieve the precise preoperative diagnosis of gastric schwannomas.

In our case, the schwannoma was discovered incidentally by abdominal CT, suggesting GIST as a likely diagnosis. Gastroscopy and EUS provided the same primary diagnosis. The tumor was misdiagnosed as a GIST until the immunohistochemical findings and mutational analysis were revealed.

Gastric schwannomas are almost uniformly benign without recurrence or metastasis, and no malignant variant was found in previous follow-up studies[6,8]. The optimal treatment for gastric schwannoma is surgical resection, which should follow the same principles with GISTs[23].

However, in recent years, therapies for gastric submucosal tumor resection have rapidly developed, and less invasive endoscopic techniques, such as snare polypectomy, endoscopic submucosal dissection, and endoscopic full-thickness resection (EFTR), have been considered and used more often. Zhai *et al*[24] conducted a 5-year retrospective study in consecutive patients who underwent endoscopic resection for gastric schwannoma at a large tertiary center, and the results indicated that endoscopic resection was effective and safe for patients with gastric schwannoma with favorable long-term outcomes. Jain *et al*[25] reported a systematic review of EFTR techniques for gastric tumors that originate from the muscularis propria and concluded that EFTR has a high success rate and low complication rate, which was a minimally invasive technique for gastric submucosal tumors.

In our case, the gastric schwannoma was treated by EFTR. To close the gastric perforation, endoscopic purse-string suture was performed. The patient had an uneventful recovery with no major complications.

CONCLUSION

Gastric schwannomas are relatively rare. Even with endoscopy and modern imaging modalities, the precise preoperative diagnosis of gastric schwannomas remains difficult. The final diagnosis of schwannoma is based on pathological and immunohistochemical examination. Gastric schwannomas are almost always benign, and patients with this type of tumor often have a favorable prognosis. Surgical resection is the optimal treatment for gastric schwannoma. Recently, minimally invasive techniques such as EFTR have been more widely considered and employed and are a safe and feasible treatment for gastric schwannomas.

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LETTER TO THE EDITOR

Gastrointestinal cytomegalovirus disease secondary to measles in an immunocompetent infant

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Abstract

Yang *et al* reported an immunocompetent infant with gastrointestinal cytomegalovirus disease secondary to measles infection. We express our opinion about the diagnosis and treatment of this rare disease.

Key Words: Gastrointestinal cytomegalovirus disease; Measles; Diarrhea; Immunocompetent infant; Rare disease

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Core Tip: We want to discuss the diagnosis and treatment issues in the rare gastrointestinal cytomegalovirus disease secondary to measles infection.

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TO THE EDITOR

We read with interest the study by Yang *et al*[1]. They highlighted the differential diagnosis and pathological features of gastrointestinal cytomegalovirus (CMV) infection in a 9-mo-old boy. In our opinion, some concepts about the diagnosis and treatment should be emphasized.

Measles leads to the morbidity of diarrhea and may cause dehydration and secondary malnutrition[2]. Its incidence is about 8%[3]. Instead, uncontrolled diarrhea caused by postnatally infected CMV in immunocompetent infants has been rarely reported. Differential diagnosis of the diarrhea cause is a great challenge to pediatric physicians, especially when most infants with neither endoscopic exam nor pathological confirmation of gastrointestinal CMV infection.

To our knowledge, most measles-infected patients only need supportive management, including fluid supply, antipyretics, and prevention of superimposed bacterial infections. There is no specific antiviral therapy. Although the efficacy in preventing and treating CMV infection has been proven in transplant recipients, Ganciclovir has not been supported effectively in treating pediatric patients[4]. It has been administered to infants with congenital infection[5] and cholestasis[6]; however, there are no controlled studies to support its effectiveness[5]. Fortunately, this 9-mo-old boy recovered completely after intravenous Ganciclovir administration with no evident side effects.

Low serum vitamin A level has been a common situation among children, even in some developed countries, *e.g.*, United States. Significant lower levels were encountered in critically ill children. Vitamin A deficit hinders the recovery course and increases measles-related complications. Besides, measles infection would further deteriorate the deficit of vitamin A serum concentration and aggravate the severity of xerophthalmia[7]. In a randomized controlled trial, lower morbidities and mortality have been found in measles-infected children after vitamin A supplement[8]. Thus, the World Health Organization recommended vitamin A administration to all acute measles-infected children[9]. We also suggest the same management to this 9-mo-old boy during the treatment course.

Vaccination is the most effective strategy to interrupt this virus transmission because it could lead to herd immunity, which must be maintained above 85% to 95% [10]. Thus, encouragement of measles vaccination is essential to avoid the occurrence of similar episodes.

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FRONTIER

Liver dysfunction and SARS-CoV-2 infection

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Abstract

Severe acute respiratory syndrome coronavirus 2 infection is the cause of coronavirus disease 2019 (COVID-19), which predominantly affects the respiratory system; it also causes systemic and multi-organic disease. Liver damage is among the main extrapulmonary manifestations. COVID-19-associated liver injury is defined as any liver damage occurring during the disease course and treatment of COVID-19 in patients with or without pre-existing liver disease, and occurs in approximately one in five patients. Abnormal liver test results have been associated with a more severe course of COVID-19 and other complications, including death. Mechanisms linking COVID-19 to liver injury are diverse. Particular consideration should be made for patients with pre-existing liver disease, such as metabolic dysfunction-associated fatty liver disease, chronic liver disease due to viral or autoimmune disease, liver transplant carriers, or cirrhosis, given the risk for more severe outcomes. This manuscript summarizes the current lines of evidence on COVID-19-associated liver injury regarding pathophysiology, clinical significance, and management in both patients with or without pre-existing liver disease, to facilitate clinicians' access to updated information and patient care. Finally, we mention the ideas and recommendations to be considered for future research.

Key Words: SARS-CoV-2; Coronavirus; COVID-19; Liver; Liver diseases; Liver failure; Liver injury; Cirrhosis

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Core Tip: Coronavirus disease 2019 (COVID-19)-associated liver injury is defined as any liver damage occurring during the disease course and treatment of COVID-19 in patients with or without pre-existing liver disease, with an observed ratio of 1:5. The presence of abnormal liver biochemical parameters has been associated with a severe course of severe acute respiratory syndrome coronavirus 2 infection and other complications, including death. Pathophysiology of COVID-19-induced liver injury is complex. Also, special consideration should be made in patients with pre-existing liver disease, such as metabolic dysfunction-associated fatty liver disease, chronic liver disease due to viral or autoimmune disease, liver transplant carriers, or cirrhosis.

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INTRODUCTION

In December 2019, multiple cases of unexplained pneumonia were reported in Wuhan, China[1]. The etiology of the outbreak was attributed to a newly identified coronavirus, initially named '2019-nCoV' (human), and subsequently renamed as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The disease was denominated coronavirus disease 2019 (COVID-19) by the World Health Organization (WHO)[1,2]. Due to the constantly increasing number of cases worldwide, on March 11, 2020, the WHO formally declared the COVID-19 outbreak as a pandemic[3]. More than a year after its appearance, SARS-CoV-2 has infected almost 10 million people worldwide and caused more than 2 million deaths[4].

Coronaviruses are members of the subfamily Coronavirinae in the family Coronaviridae and the order Nidovirales (International Committee on Taxonomy of Viruses). This subfamily consists of four genera (*Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*)[5]. The *Betacoronavirus* include the Middle East respiratory syndrome (MERS) coronavirus, SARS-CoV, and SARS-CoV-2. These viruses have a positive-sense single-stranded RNA genome[6]. The angiotensin-converting enzyme 2 (ACE2) has been identified as the main viral receptor for SARS-CoV and SARS-CoV-2[5,7]. ACE2 is ubiquitously and widely expressed in many organs and systems, including the lungs, cardiovascular system, kidneys, pancreas, intestines, liver, adipose tissue, and muscular and nervous systems[8]. Another cellular protein, the transmembrane protease serine 2 (*i.e.*, TMPRSS2), facilitates viral entry into the host cells through plasma membrane surface interaction[9].

SARS-CoV-2 could be transmitted from person to person through close contact, respiratory droplets, and aerosol[10]. The manifestations of COVID-19 represent a wide clinical spectrum, which ranges from asymptomatic individuals or mild respiratory symptoms to severe-critical illness; overall, it is categorized as a mild, severe, or critical illness[11]. Although SARS-CoV-2 predominantly causes respiratory symptoms, it can also result in extrapulmonary disease, including thrombotic complications, myocardial damage, acute kidney failure, gastrointestinal symptoms, hepatocellular injury, hyperglycemia and ketosis, neurologic illnesses, ocular symptoms, and dermatologic manifestations[12,13]. These manifestations can occur in subjects without identified pre-existing organic disease, as well as in individuals with comorbidities, such as patients with hypertension, obesity, and chronic liver disease, among others.

The objective of this review is to discuss and show current data regarding liver dysfunction caused by SARS-CoV-2 infection in patients with or without pre-existing liver disease, its pathophysiology and management, as well as the prospects for future research.

SARS-COV-2 INFECTION AND LIVER DYSFUNCTION IN PATIENTS WITH NO PREVIOUS LIVER DISEASE

Epidemiology

COVID-19-associated liver injury is defined as any liver damage occurring during disease course and treatment of COVID-19 in patients with or without pre-existing liver disease[14]. A summary of the principal studies about liver damage in COVID-19 patients is showed in Table 1. Studies have shown that one in five patients with COVID-19 develop abnormalities in liver function tests[15]. A large systematic review that included 64 studies with 11245 patients with SARS-CoV-2 infection showed the following prevalence of abnormal liver function parameters: Elevated aspartate aminotransferase (AST) in 23.2%; alanine aminotransferase (ALT) in 21.2%; elevated total bilirubin in 9.7%; increased gamma-glutamyltransferase (GGT) in 15.0%; and increased alkaline phosphatase in 4.0%[16]. The presentation of liver injury during COVID-19 infection occurs mostly during the acute hospitalization period and it is associated with increased length of hospital stay, worse pulmonary score on computed tomography (commonly referred to as CT), overall severity of disease, and increased mortality.

In a single-center retrospective study that described temporal variations of liver injury during hospitalization due to SARS-CoV-2 infection, the percent of subjects with elevated aminotransferases (transaminitis) in mild cases was 12.6% *vs* 46.2% in severe cases. Most of the patients presented ALT elevations between days 4 and 17 of their hospitalization, with a mean of 10.7 d and 7.3 d in mild and severe cases, respectively. During treatment, increases in liver function test parameters were predominantly mild and elevations in ALT and AST were largely isolated, occurring in 19% of patients. The majority of patients were discharged with normal liver function parameters[17]. A large retrospective multicenter cohort study that included 5771 patients with COVID-19 pneumonia determined the distribution and temporal patterns of liver injury indicators in these patients; an initial elevation of AST, followed by ALT in severe patients, and mild fluctuation in total bilirubin levels in both non-severe and severe disease were found[18]. Another study of 79 in-patients with COVID-19 found that the extent of pulmonary lesions observed on CT was predictive of liver function damage [19]. In a systematic review that included 45 studies, abnormal liver biochemical indicators were detected at admission in 27.2% of cases, which increased to 36% during hospitalization, and there was a higher incidence of severe and/or critical cases [20]. Another meta-analysis revealed that, among 15407 patients with SARS-CoV-2 infection, the incidence of elevated liver chemistries was 23.1% at early presentation and 24.4% throughout the course of illness[21]. A prospective cohort study in 1611 hospitalized patients from 11 Latin American countries found abnormal liver tests on admission in 45.2% and that such was independently associated with death [odds ratio (OR): 1.5, 95% confidence interval (CI): 1.1-2.0] and severe COVID-19 (OR: 2.6, 95%CI: 2.0-3.3)[22]. A systematic review of 24 studies (5961 subjects) found that, among COVID-19 patients who were critically ill, the OR of hypoalbuminemia was 7.1, of AST elevation was 3.4, of ALT elevation was 2.5, and of hyperbilirubinemia was 1.7[23]. Systematic reviews with meta-analyses showed that patients with prolonged prothrombin time had a higher odds for progression to severe disease (OR: 1.82) and intensive care unit (ICU) admission (OR: 2.18)[24,25]. A synthesis of the literature that compared survivors and non-survivors with severe COVID-19 patients showed an OR of 1.98 (95%CI: 1.39-2.82) for liver dysfunction and mortality[26]. Similarly, previous investigations have shown that liver injury was common among patients infected by SARS-CoV and MERS coronavirus, and associated with the severity of diseases[27].

In patients with SARS-CoV-2 infection, the degree of transaminitis is generally mild [22,23], defined as less than 5 times the upper reference limit, and severe liver failure occurs infrequently[28]. In a cohort of 5700 patients from New York, United States, AST and ALT were both commonly increased (58.4% and 39.0% of subjects, respectively). In this same study, 56 (2.1%) patients had developed severe acute liver injury (defined as an increase in ALT or AST of > 15 times the upper limit of normal) and an association with mortality was found in 95%[29]. Finally, abnormal liver function test has been observed in patients with subclinical disease (elevated AST in 8.7% and elevated ALT in 8.9%)[30].

Pathophysiology

The mechanisms of liver injury in patients with SARS-CoV-2 infection are diverse. It has been postulated that SARS-CoV-2 may cause cytopathic effects due to viral replication after entrance into the liver and bile duct cells *via* interaction with ACE2

Table 1 Principal studies about liver damage in coronavirus disease 2019 patients

Ref.	Study	Findings
Mao *et al*[15]	SR (35 studies, *n* = 6686)	The prevalence of abnormal liver functions was 19% (CI: 9-32). Patients with severe COVID-19 had higher rates of abnormal liver function including increased ALT (OR: 1.89, CI: 1·30-2·76) and increased AST (OR: 3.08, CI: 2.14-4.42) compared with those with non-severe disease
Wijarnpreecha *et al*[16]	SR (64 studies, *n* = 11245)	The prevalence of elevated AST, ALT, total bilirubin, GGT, and alkaline phosphatase was 23.2%, 21.2%, 9.7%, 15.0%, and 4.0%, respectively. The prevalence of elevated AST was higher among those with severe cases (45.5%) compared to non-severe cases (15.0%). Co-existing CLD presented in up to 37.6% of patients with COVID-19
Wang *et al*[17]	Single-center retrospective study (*n* = 105)	Fifty-six percent of the patients had abnormal ALT, AST, or total bilirubin during the illness (91.4% cases were ≤ 3 fold of the ULN). The percentage of patients with elevated both ALT and AST was 12.7% in mild cases *vs* 46.2% in severe cases. One third of patients with severe disease started to have abnormal ALT after admission, and 73.3% of all patients had normal ALT before discharge
Lei *et al*[18]	Multicenter retrospective cohort study (*n* = 5771)	The distributional and temporal patterns of liver injury indicators were following: AST elevated first, followed by ALT, in severe patients. Alkaline phosphatase modestly increased during hospitalization and largely remained in the normal range. The fluctuation in total bilirubin levels was mild in the non-severe and severe groups
Xie *et al*[19]	Retrospective study (*n* = 79)	Logistic regression analyses suggested that the extent of pulmonary lesions on CT was a predictor of liver function damage
Wu *et al*[20]	SR (45 studies, *n* = 7228)	The incidence of any abnormal liver biochemical indicator at admission and during hospitalization was 27.2% and 36%, respectively
Kulkarni *et al* [21]	SR (107 studies, *n* = 20874)	The prevalence of CLD was 3.6% (CI: 2.5-5.1). The incidence of elevated liver chemistries was 23.1% (CI: 19.3-27.3) at initial presentation and 24.4% (CI: 13.5-40) during the illness. The incidence of DILI was 25.4% (CI: 14.2-41.4). The prevalence of CLD among 1587 severely infected patients was 3.9% (3%-5.2%). CLD was not associated with the developing severe COVID-19 (OR: 0.81, CI: 0.31-2.09) compared to non-CLD patients. COVID-19 patients with elevated liver chemistries had an increased risk of mortality (OR: 3.46 CI: 2.42-4.95) and severe disease (OR: 2.87, CI: 2.29-3.6) compared to patients without
Mendizabal *et al*[22]	Multicenter prospective cohort study (*n* = 1611)	Abnormal liver tests on admission were present on 45.2% and were independently associated with death (OR: 1.5, CI: 1.1-2.0), and severe COVID-19 (OR: 2.6, CI: 2.0-3.3). The prevalence of CLD was 8.5%
Wong *et al*[23]	SR (24 studies, *n* = 5961)	In subjects with critical COVID-19, the OR of hypoalbuminemia was 7.1 (CI: 2.1-24.1), of AST elevation was 3.4 (CI: 2.3-5.0), of ALT elevation was 2.5 (CI: 1.6-3.7), and of hyperbilirubinemia was 1.7 (CI: 1.2-2.5)
Zhu *et al*[24]	SR (34 studies, *n* = 6492)	Patients with severe COVID-19 showed significantly longer PT, and a longer PT was associated with a higher risk to die
Elshazli *et al* [25]	SR (52 studies, *n* = 6320)	Prolonged PT was associated with a higher risk of progression to severe COVID-19 (OR: 1.82) and ICU admission (OR: 2.18)
Wu and Yang [26]	SR (13 studies, *n* = 3722)	The comparison between survivors and non-survivors with severe COVID-19 patients showed an OR of 1.98 (CI: 1.39-2.82) for liver dysfunction and mortality
Richardson *et al* [29]	Multicenter prospective cohort study (*n* = 5700)	In hospitalized COVID-19 patients, AST and ALT were both commonly increased (58.4% and 39.0% of patients, respectively). Fifty-six (2.1%) subjects developed a severe acute liver injury with a mortality of 95%
Shi *et al*[30]	Two-center retrospective study (*n* = 81)	Abnormal liver function test was found in patients with subclinical disease (elevated AST in 8.7% and elevated ALT in 8.9%
Sultan *et al*[58]	SR (47 studies, *n* = 10980)	The prevalence estimates of elevated liver abnormalities were as follows: AST 15.0% (CI: 13.6-16.5), ALT 15.0% (CI: 13.6-16.4), and abnormal bilirubin 16.7% (CI: 15.0-18.5)

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CI: Confidence interval; CLD: Chronic liver disease; COVID-19: Coronavirus disease 2019; CT: Computed tomography; DILI: Drug-induced liver injury; GGT: Gamma-glutamyltransferase; ICU: Intensive care unit; PT: Prothrombin time; OR: Odds ratio; SR: Systematic review; ULN: Upper limit of normal.

and TMPRSS2[31]. ACE2 expression is considerably higher in cholangiocytes (59.7%) than in hepatocytes (2.6%)[32]. Cholangiocytes have an important role in immune response, inflammation, and liver regeneration[33]. Furthermore, the expression of ACE2 in hepatocytes increases in cases of liver injury[34]. In postmortem liver biopsies from two patients who died from COVID-19, typical coronavirus particles were identified in the cytoplasm of hepatocytes, with cytopathic damage characterized by mitochondrial swelling, endoplasmic reticulum dilatation, and glycogen granule decrease[35]. These findings support the hypothesis of virus-related hepatic damage. However, other liver biopsy specimens of a patient who died from COVID-19 showed moderate microvesicular steatosis and mild lobular and portal activity, which are not specific and could have been caused by the viral infection, drug-induced liver injury (DILI), or nonalcoholic fatty liver disease (NAFLD)[36,37]. In addition, viral inclusion

bodies were not detected in liver tissue[37]. Another postmortem liver histopathologic study also reported microvesicular steatosis, accompanied by overactivation of T cells, suggesting a component of immune-mediated liver injury[38]. SARS-CoV-2 could also cause liver damage through the generation of endothelitis[39]. Endothelial cells are involved in ischemia-reperfusion liver damage and promote oxidative stress through reactive oxygen species and derivatives of nitric oxide[40]. Post-mortem wedge liver biopsies from 48 patients who died from severe COVID-19 disease showed vascular alterations characterized by an increased number of portal vein branches associated with massive lumen dilatation, partial or complete luminal thrombosis of portal and sinusoidal vessels, and marked focal enlargement and fibrosis of the portal tract[41]. In addition, transaminitis has been reported in some cases of portal thrombosis due to SARS-CoV-2 infection[42,43].

The immune overactivation associated with SARS-CoV-2 infection may also be involved in liver injury. Prominent elevations in serum inflammatory cytokine levels, such as interferon-γ, interleukin (IL)-1β, IL-6, IL-10, soluble IL-2 receptor α, and tumor necrosis factor, are present in patients with COVID-19, especially those with severe pneumonia[44,45]. This can lead to immune-mediated liver injury *via* activation of intrahepatic CD4+ and CD8+ cells, T cells, Kupffer cells, and a dysregulated innate immune response[46,47]. This phenomenon has also been described in infections caused by herpes viruses (Epstein-Barr virus, cytomegalovirus, and herpes simplex virus), parvovirus, adenovirus, and SARS-CoV[47]. Moreover, COVID-19 patients with increased AST also have elevated IL-6, ferritin, lactate dehydrogenase, and C-reactive protein compared to patients with normal AST[48].

In the course of infection by SARS-CoV-2, hepatic ischemia and hypoxia with impaired tissue perfusion can develop as a consequence of pneumonia-associated hypoxemia, circulatory failure, respiratory distress syndrome, and multiple organ failure[49]. Hepatic congestion secondary to high positive end-respiratory pressure in mechanically-ventilated patients may also enhance the degree of hypoxic damage in hepatocytes[32,46].

Liver injury associated with COVID-19 may also occur secondary to the potentially hepatotoxic effects of many drugs used for its treatment, such as acetaminophen, antivirals, antibiotics, corticosteroids, and immune modulators, among others. The presence of microvesicular steatosis and liver inflammation in liver biopsies of patients with SARS-CoV-2 infection could also be drug-related[37]. The drug-cytochrome P-450 interaction could explain some of the liver toxicity secondary to such drugs as azithromycin, lopinavir/ritonavir, hydroxychloroquine, and acetaminophen[50]. Additionally, patients with underlying NAFLD might be more susceptible to DILI because the cytokine monocyte chemoattractant protein-1 (*i.e.*, MCP-1) is often elevated in COVID-19 patients and could exacerbate steatohepatitis[51]. In a systematic review which included 107 articles (n = 20874 patients), the pooled incidence of DILI in COVID-19 patients was 25.4%[21]. A more detailed description of the drugs to treat SARS-CoV-2 infection and their potential risk of liver damage is discussed later.

SARS-CoV-2 RNA has been detected in feces, and it appears plausible that virus and inflammatory mediators present within the gut lumen could reach the liver through the portal circulation. Kupffer cells could attempt to clear the viral particles, consequently increasing the inflammatory response[39,50].

Other causes that are not necessarily associated with direct hepatocyte injury may explain the abnormal liver biochemical indicators in patients with SARS-CoV-2 infection. Transaminitis could originate from myositis rather than liver damage[52]. Muscular injury [defined as the presence of myalgias and creatinine kinase (CK) > 200 U/L] has been documented in 10% of hospitalized patients by COVID-19 and some studies have reported increased levels of myoglobin of CK in association with COVID-19 severity[46,53,54]. Hypoalbuminemia could be explained by decreased hepatic synthesis, malnutrition, increased catabolism, and albumin extravasation because of increased capillary permeability[55,56]; we must recall that hypoalbuminemia is also an acute phase reactant. Alkaline phosphatase and GGT are considered as cholangiocyte-related enzymes, but the higher prevalence of abnormal GGT may be attributed to acute inflammatory stress because the GGT is recognized as a surrogate marker for increased oxidative stress and inflammation[57].

Management

The recommendations by the American Gastroenterology Association and the World Gastroenterology Organization regarding the general approach to patients with SARS-CoV-2 infection and liver injury are as follows[58,59]: (1) In patients with abnormal liver function test results in the context of suspected or known COVID-19, evaluate for

alternative etiologies, including proof of viral hepatitis, particularly in developing countries; (2) Routine outpatient testing of liver biochemistries is not recommended; (3) In in-patients with COVID-19, obtain baseline liver indicators at the time of admission and consider its monitoring throughout the hospitalization; and (4) Avoid routine liver imaging, unless it will alter management.

FATTY LIVER DISEASE

General implications and epidemiology

The presence of metabolic dysfunction-associated fatty liver disease (MAFLD; previously known as NAFLD)[60] in the patients with infection by SARS-CoV-2 (*i.e.*, COVID-19) is important given that specific metabolic and cardiovascular comorbidities intrinsically related to MAFLD, like hypertension, diabetes, obesity, coronary artery disease, and cerebrovascular disease, were identified as independent risk factors associated with increased risk of infection by SARS-CoV-2[61,62], especially hypertension[52], diabetes[63,64] , and obesity [body mass index (BMI) > 30 kg/m^2][65]; furthermore, morbid obesity (BMI > 40 kg/m^2) is a strong risk predictor of hospitalization in patients with COVID-19[66].

MAFLD has been associated with an increased risk for mortality in patients with community-acquired pneumonia, which is further enhanced in patients with advanced liver fibrosis[67]. Also, MAFLD has been associated with an increased risk for bacterial infections, independent of the presence of metabolic syndrome and especially among patients with vitamin D deficiency[68]. The relevance of this is the recognition of MAFLD as a risk factor for severe infections.

MAFLD is an independent risk factor for progression of COVID-19 respiratory disease (OR: 6.4, 95%CI: 1.5-31.2), and this risk is heightened in patients with associated liver fibrosis[38,69,70]. In addition, MAFLD is associated with a higher likelihood of abnormal levels of aminotransferases at time of discharge as well as increased duration of virus shedding, which renders the individual infectious for 5 d longer[38,59]. The increased risk for viral infection in patients with MAFLD may be related to the pre-existent intrinsic up-regulation of ACE2 receptors that occurs in this disease, as well as in liver injury; in addition, the ACE2 receptors have been identified as the cellular point of entry of SARS-CoV-2[59].

A multicenter study of COVID-19 patients in the United States found a significant association between MAFLD and ICU admissions (OR: 2.30, 95%CI: 1.27-4.17, P = 0.03) as well as need for mechanical ventilation (OR: 2.15, 95%CI: 1.18-3.91, P = 0.02) but did not find a correlation with increased mortality[71]. A cohort study in the United Kingdom (Forlano *et al*[72]) showed that patients with MAFLD were younger than their counterparts without MAFLD. MAFLD *per se* had no direct correlation with increased mortality; however, among those who died in hospital, the risk was associated with male sex (71% *vs* survivors: 50%, P = 0.01), elevated ferritin (2076 μg/L *vs* survivors: 688 μg/L, P = 0.003), and early weaning score (n = 7 *vs* survivors: 3, P = 0.047). A recent systematic review of eight studies, including 8142 patients with COVID-19 and 833 of those with MAFLD, found that MAFLD by itself conferred an increased risk for severe COVID-19 of 2-fold (OR: 2.358, 95%CI: 1.902-2.923, P < 0.001) [73]. Finally, a meta-analysis of six studies (n = 1293) found an increase in the risk of COVID-19 disease severity of almost 3-fold (OR: 2.93, 95%CI: 1.87-4.60, I^2 = 34.3%, P = 0.166) among patients with MAFLD[74].

The comorbidities and increased inflammatory state in patients with MAFLD confer a hypothetical increased risk for DILI and, hence, careful monitoring of liver function is warranted in these patients, as are efforts to minimize exposure to polypharmacy [75].

Age and MAFLD

A cohort study in the United Kingdom found that most patients with COVID-19 and MAFLD were younger than 60 years old, as compared with patients with no MAFLD [72]. Among younger patients (age < 60 years old), the risk of severe COVID-19 is increased by 4-fold among those with concomitant NAFLD (OR: 4.07, 95%CI: 1.20-13.79, P = 0.02)[76,77].

Histopathologic changes in COVID and MAFLD

Although the severity of hepatic visceral fat correlates with the risk of COVID-19 infection[78], in general, the histopathologic findings in the liver in patients with SARS-CoV-2 infection have been presumed to be related mostly to the underlying liver

disease (*e.g.*, MAFLD) or other comorbidities (*e.g.*, drug toxicity and ICU care) rather than to a direct effect of the viral infection[79]. However, Nardo *et al*[80] described several mechanisms in which there is increased liver steatosis as a consequence of the viral infection; these include impaired mitochondrial dysfunction, endoplasmic reticulum stress-induced lipogenesis, and inflammation (including cytokine storm) with increased IL-6 and hyperstimulation of the mammalian target of rapamycin (*i.e.*, mTOR). The mTOR is also activated by glucose and insulin, and insulin resistance is also intrinsically associated with MAFLD; therefore, not only is there already an underlying inflammatory state but it can also be enhanced further by direct viral cytopathic effect[80].

Obesity and MAFLD

When considering the correlation of obesity and metabolic disease with the increased risk of COVID-19 as well as of severity of clinical presentation, one of the most accepted hypotheses is the presence of underlying chronic inflammatory state in these patients enhancing oxidative stress and increasing atherosclerosis and cardiovascular disease[81,82]. In addition, it is well evidenced that obesity confers an impaired immune response to viruses, with associated prolonged viral shedding as well as emergence of virulent minor variants[83]. If the readers would like to explore more intricate descriptions of the pathophysiology of inflammation in MAFLD and obesity, they are referred to the excellent manuscript by Portincasa *et al*[84].

In a study conducted in a Chinese population by Gao *et al*[65], the presence of obesity was found to increase the risk of severe COVID-19 by almost 3-fold (OR: 2.91, 95%CI: 1.31-6.47); furthermore, this risk was incrementally raised by 12% per unit of increase in BMI (OR: 1.12, 95%CI: 1.01-1.23). A prospective study of 5279 patients admitted to a hospital in New York, United States found that BMI > 40 kg/m^2 increased the risk of hospitalization by more than 2-fold (OR: 2.5, 95%CI: 1.8-3.4) and the risk of critical illness by 50% (OR: 1.5, 95%CI: 1.0-2.2)[66]. A very important epidemiological risk factor was reported by Kass *et al*[85], who identified a negative correlation of increased BMI and age among patients with severe COVID-19 infection, which showcases its impact in young patients. The co-existence of obesity and MAFLD has also been associated with an almost 6-fold increase in the risk of severe COVID-19 infection[38,86]. Furthermore, the severity of steatosis also correlates with the risk of infection as demonstrated by Roca-Fernández *et al*[78], who reported that among obese patients (BMI > 30 kg/m^2) with liver fat > 10%, the risk of symptomatic COVID-19 infection was increased almost 3-fold (OR: 2.96, 95%CI: 1.12-7.78, P = 0.02).

Management of patients with MAFLD in the era of COVID-19

The World Gastroenterology Organization recently published its recommendations for management of patients with MAFLD in the COVID-19 era, which essentially recommends to[59]: (1) Recognize the presence of MAFLD in patients with underlying metabolic disease, formally identifying its stage and grade; (2) Recognize that obesity and diabetes mellitus increase the risk of mortality from respiratory illnesses, including COVID-19; (3) Recognize that the risk of respiratory disease progression is higher in patients with MAFLD; and (4) Encourage patients with MAFLD to make lifestyle changes that will mitigate risk factors (*e.g.*, obesity) that can worsen the prognosis of COVID-19.

SARS-COV-2 INFECTION IN LIVER TRANSPLANT PATIENTS

In this section, we will focus on the assessment and management of patients with a transplanted liver who present with infection by SARS-CoV-2 (COVID-19).

Liver transplant patients are frail and have many risk factors for COVID-19 infection, including immunosuppression, in addition to other underlying comorbidities[87]. The symptomatology among patients with solid organ transplant who are infected with COVID-19 is similar to that among the general population; however, the severity and outcomes are worse, especially as both are impacted by their comorbidities[88,89].

Epidemiology

Imam *et al*[87] reported a review of ten studies from all over the world that included 22 patients with orthotopic liver transplant, among which 72% experienced clinical recovery from COVID-19, with a median duration of illness of 17 d. ICU admission was required in 28.6% of patients and the mortality rate in the cohort was 13.6%. On

the other hand, a European liver transplant cohort study of 57 patients with COVID-19 (70% male; median age of 65 years) found no significant impact of decreasing immunosuppression (37% of patients). The rate of hospitalization was 72%, and acute respiratory distress syndrome was present in 19% of cases. The overall mortality in the cohort was 12%, which increased to 17% among hospitalized patients. Among those who died, a history of cancer was common (5 out of 7 patients)[90]. An international multicenter cohort study of 151 adult liver transplant recipients from 18 countries (68% male; median age of 60 years) performed a comparison with 627 patients without a history of liver transplant (52% male; median age of 73 years). The liver transplant cohort had more frequent rates of ICU admission (28% *vs* 8%, $P < 0.0001$) and invasive ventilation (20% *vs* 5%, $P < 0.0001$). The mortality rate was 19% in the liver transplant cohort *vs* 27% in the comparison cohort ($P = 0.046$). After adjusting for comorbidities (age, sex, creatinine concentration, obesity, hypertension, diabetes, and ethnicity), liver transplantation was not associated with a significant increase in the risk of mortality in patients with COVID-19; however, multivariable logistic regression analysis demonstrated that the mortality increase in liver transplant patients was associated with age [(OR: 1.06, 95%CI: 1.01-1.11) per 1 year increase], serum creatinine [(OR: 1.57, 95%CI: 1.05-2.36) per 1 mg/dL increase], and cancer (OR: 18.30, 95%CI: 1.96-170.75) [91].

Recommendations for management of liver transplant patients with COVID-19

Multiple guidelines and reviews have been published with the aim of outlining the management of patients with COVID-19 who are either liver transplant candidates or have post-liver transplant status[92-98]. Most have very similar recommendations to the ones by the American Association for the Study of Liver Diseases (AASLD)[99] and Asian-Pacific Association for the Study of the Liver (APASL)[100] summarized below.

The AASLD published an Expert Panel Consensus Statement for Management of Liver Transplant During the COVID-19 Pandemic[99].

Recommendations that apply to the patient post-transplant status: (1) Given the associated high risk for severe COVID-19, these patients must be prioritized for testing; (2) In patients with COVID-19 and elevated aminotransferases, other etiologies unrelated to COVID-19 should be considered, such as viral hepatitis, myositis (especially if AST > ALT), cytokine release syndrome, and ischemia; (3) Ancillary studies should be minimized (*e.g.*, ultrasound and magnetic resonance imaging) to avoid the risk of healthcare personnel exposure, unless it will change management (*e.g.*, venous thrombosis and biliary obstruction); and (4) In the post-transplant time, which includes concerns for acute cellular rejection, a formal histopathologic confirmation with biopsy is necessary.

In patients who are candidates for transplantation: (1) The pandemic may affect the waiting time to transplant. Care teams must consider the evaluation of patients with a high model for end-stage liver disease score or hepatocellular carcinoma with severe disease (upper levels of Milan criteria), who would have a higher priority; (2) Screening for COVID-19 must be done on both the donor and the recipient. At this time, donors who are positive for SARS-CoV-2 are not considered eligible for organ donation. In the same tenure, transplantation is not recommended for COVID-19-positive patients; (3) Care teams should aim to select donor livers with a low risk of delayed graft function, in order to avoid complications and duration of postoperative hospitalization; and (4) Care teams may consider postponing a liver donor program during the pandemic.

In post-transplant patients with COVID-19 infection: (1) It is adequate to consider decreasing the dosage of high-dose prednisone. Although, a dosage that is sufficient to avoid adrenal insufficiency must be maintained; and (2) Reduction of azathioprine, mycophenolate, or daily calcineurin inhibitor dosages can be considered, especially in the setting of lymphopenia, fever, or worsening pneumonia attributed to COVID-19.

Very similar recommendations have been published by the APASL[100]. In addition, they recommend immunization of all patients with liver transplant against pneumococcus and influenza. Other recommendations include avoiding drugs that would have a significant impact on the tacrolimus levels, such as would occur in any other clinical setting[98].

One of the considerations to keep in mind for patients with liver transplant who become infected with COVID-19 is their public health impact, given their risk to be long-term carriers not only due to the slower clearance of the virus but also as they can be asymptomatic carriers[96]. This increases their risk for viral spread in the community, as well as nosocomially as they may have prolonged hospitalizations due

to their medical complexity[96].

Conclusions

Patients with liver transplant must be managed with similar protocols as non-transplanted patients; yet, clinicians must be mindful of the impact of immunosuppression on these patients' viral shedding and carrier status, as well as of medication interaction.

COVID-19 AND LIVER CIRRHOSIS

General considerations and epidemiology

The current evidence that describes the overall impact of COVID-19 in patients with liver cirrhosis, either compensated or decompensated, is scant. However, extrapolating from the current knowledge of the physiopathology of both diseases, the expected morbidity and mortality are more severe when compared to other groups. Many factors must be considered in the interaction of COVID-19 and the liver; for instance, most of the drugs used in the treatment of COVID-19, including biologic agents, can have either a direct hepatotoxic effect or reactivate chronic viral diseases, such as hepatitis B virus[14]. Other studies have detected the presence of SARS-CoV-2 in the liver tissues of patients who had died from COVID-19[101], suggesting viral replication at this level. In patients with liver cirrhosis, both effects have a critical impact as they may worsen the course of the disease by damaging the remaining liver parenchyma[96,102]. Otherwise, there are studies with findings suggesting that if the liver damage induced by COVID-19 is immunologically driven, then the immunocompromised status of cirrhotic patients might be more protective than harmful[103]. However, due to the limited number of patients with chronic liver disease within individual studies on COVID-19 to date, the true impact of underlying liver disease on viral progression and outcomes is unknown.

Existing evidence about outcomes of COVI-19 infection in patients with chronic liver disease is contradictory. A pooled analysis of six studies estimating the impact of chronic liver disease in COVID-19 patients suggested that chronic liver disease and cirrhosis seem to play a minor role in determining patient progression towards the severe forms of the disease; in that study, there was no correlation found between chronic liver disease and increased odds of the severe form of COVID-19 (OR: 0.96, 95%CI: 0.36-2.52) nor with increased odds of mortality (OR: 2.33, 95%CI: 0.77-7.04) [104]. Similar data were reported by Bangash *et al*[46]; specifically, a mortality rate of 0 to 2% was shown by COVID-19 patients with liver cirrhosis. A study of 22 patients with chronic liver disease, among which only three had liver cirrhosis, found that the only significant difference between patients with chronic liver diseases *vs* those without was the risk of progression to severe forms of COVID-19 ($P < 0.001$); however, there were no statistical differences in other variables, such as in-hospital days, death/discharge, or significant changes in liver enzyme values[69]. Finally, a meta-analysis found that the pooled prevalence of chronic liver disease among studies reporting on severity of COVID-19 was 2.64% (95%CI: 1.73-4.00), with 3.03% (95%CI: 1.97-4.64) among severe and 2.20% (95%CI: 1.16 - -4-15) among non-severe COVID-19. The relative risk of chronic liver disease in severe *vs* non-severe patients was 1.69 (95%CI: 1.05-2.73)[105].

The controversy in the data involves evidence generated by another meta-analysis which demonstrated that patients with a pre-existing chronic liver disease have an increased risk for severe COVID-19 (53.33%) and higher mortality (17.65%)[106]. This outcome is likely related to coexistent thrombocytopenia and lymphopenia[32,107] as well as cirrhosis-associated immune dysfunction[108]; therefore, precautions against SARS-CoV-2 infection are warranted among patients with cirrhosis. In addition, stress and sepsis related to over-imposed bacterial infections in COVID-19 are particularly risky and problematic in patients with decompensated liver cirrhosis, given the associated risk of developing acute-on-chronic liver failure, increasing the underlying risk of death from 26.2% to 63.2%; however, most of the studies have shown the cause of death in most liver cirrhosis patients with COVID-19 not to be due to progressive liver disease but rather to pulmonary disease[107,109]. Nonetheless, recent studies have found a higher 30-d mortality rate among patients with cirrhosis and COVID-19 [110], and the presence of cirrhosis has even been proposed as an independent predictor of mortality[71].

Treatment recommendations

The current available evidence suggests that COVID-19 patients with liver cirrhosis have worse outcomes and disease progression than those without. Thus, the treatment recommendations by most international associations are as follows: (1) Minimal exposure to medical staff, ideally leveraging telemedicine as the preferred method; (2) Listing for liver transplantation being restricted to patients with acute liver failure or poor short-term prognosis; (3) Prophylaxis regimens for spontaneous bacterial peritonitis and hepatic encephalopathy being strictly followed at home, to prevent decompensation and the need for hospital admissions; (4) Testing for SARS-CoV-2 for every patient with cirrhosis and acute decompensation or acute-on-chronic liver failure[95]; (5) In-person new patient visits being restricted to only those with significant liver diseases, such as jaundice, elevated transaminases > 500 U/L, or recent decompensation; (6) Rescheduling elective procedures, such as screening for varices and hepatocellular carcinoma; and (7) Urgent procedures, such as paracentesis, being performed using a COVID-19-free path in either the hospital or home care[111-113].

The data regarding vaccination against SARS-CoV-2 in patients with liver cirrhosis is scarce. Despite the inclusion of nearly 100000 participants in all the vaccination trials, data for patients with liver disease are extremely limited. For example, in the Pfizer vaccination study, 217 (0.6%) of 37706 participants had liver disease and only three (< 0.1%) had moderate to severe liver disease. Similar numbers can be seen in the Moderna trial. Importantly, criteria used to classify liver disease and its severity in each study were not specified. Therefore, the real SARS-CoV-2 vaccine safety profile and its immunological response in patients with liver cirrhosis will almost completely come from post-licensing, real-world data[114].

We must not forget the underlying deficiencies in innate and humoral immunity, termed cirrhosis-associated immune dysfunction, that are present in patients with advanced liver disease. It can be hypothesized that this may confer an attenuated immune response to vaccination, but this remains to be verified[115]. Nonetheless, taking into account the risk of COVID-19 progression in these patients (as described above) and considering that there are no absolute contraindications to SARS-CoV-2 vaccination in cirrhosis, it is fundamental to prioritize immunization in this subgroup. AASLD recommendations establish that, when the supply of COVID-19 vaccine is limited, it is reasonable to prioritize patients with higher model for end-stage liver disease and Child-Turcotte-Pugh scores for vaccination together with those who are anticipated to undergo imminent liver transplantation; ideally, however, all chronic liver disease patients should be vaccinated whenever possible[114,116,117].

MISCELLANEOUS

Autoimmune hepatitis

Treatment of autoimmune hepatitis (AIH) has posed a challenge during this COVID-19 pandemic. One of the main challenges is the management with immunosuppressive drugs, since these medications are associated with an increased risk of severe viral infections[118]. COVID-19 has been hypothesized to decompensate or increase the risk of an unfavorable course of liver disease[99]. In a small cohort in northern Italy of ten AIH patients on immunosuppressive treatment who became infected with COVID-19, five developed COVID-19 pneumonia, with only one patient dying (who had decompensated cirrhosis previously), while the rest of the patients fully recovered. Regarding the impact of the COVID-19 on AIH, only one patient presented relapse associated with the interruption of immunosuppressive treatment; it was concluded that patients with AIH under immunosuppressive and COVID-19 treatment have no increased risk of severity or complications of COVID-19 disease when compared to the general population[119]. A multicenter study that included 70 AIH patients with COVID-19, where 58 patients were on immunosuppressant therapy, and of whom 52% received combined immunosuppressant therapy, found that 65 (93%) patients reported clinical symptoms, mainly respiratory (74%) and gastrointestinal (26%), and 15% were asymptomatic. Mortality occurred in 16 (22.8%) patients; among those who died, the causes were attributed to a pulmonary etiology in nine (56%), liver etiology in five (31%), and cardiac etiology in two (13%). The factors associated with death in AIH patients were age (OR: 2.01 per 10 years, 95%CI: 1.07-3.81, P = 0.031), Child-Pugh B score (OR: 42.48, 95%CI: 4.41–409.53, P = 0.001), and Child Pugh C score (OR: 69.30, 95%CI: 2.83-1694.50, P = 0.009) unrelated to immunosuppressant use and death[120]. When comparing this group of patients with a cohort of patients with liver disease

without AIH, the authors did not find a statistical difference among groups, concluding that AIH patients on immunosuppressive therapy are not associated with an increased risk or severity of SARS-COV-2 infection; therefore, the recommendation is not to decrease or discontinue immunosuppressive treatment in patients with AIH and COVID-19, due to the risk of decompensation of liver disease.

Viral hepatitis

Hepatitis B (HB) and hepatitis C (HC) represent major global public health problems [121,122]. The coinfection of SARS-CoV-2 and HB and/or HC depends on local prevalence. For example, a Chinese study of a cohort of 1099 cases of COVID-19 patients demonstrated that 23 (2.1%) had pre-existing HB; in contrast, in the northeastern United States, a series of 5700 patients hospitalized with COVID-19 showed a prevalence of 0.1% HB and < 0.1% HC[29,52].

The impact on the evolution of COVID-19 and HB superinfection is uncertain. The first reports of the cohort in Wuhan, China found that 2.1% (23/1099) of patients with HB accounted for 0.6% of severe cases[52]. Another report from different hospitals in China involving a cohort of 571 patients showed that 15 (2.63%) patients had underlying HB; the incidence of admission to ICU and death in the HB group was 0% and 6.47% (36/556), respectively, in the non-HB group[123]. Contradictory data stem from other studies. A retrospective study of 70 patients with COVID-19 and HB documented a higher susceptibility of acquiring COVID-19, as well as higher rates of hepatic damage and coagulation disorders and severity of the disease, without having an impact on hospital stay or mortality[124]. A retrospective study of 123 patients with COVID-19, found that HB was present in 15 (12.2%) patients, among who 11 (73.3%) evolved favorably and were discharged from the hospital uneventfully; out of the four who remained in the hospital, two (13.3%) died from digestive bleeding. In comparison, the mortality rate was lower in the group of 108 patients with COVID-19 without HB, among which only eight (5.6%) remained in the hospital and three (2.8%) died due to respiratory failure[125]. Theoretically, this association of poor clinical forecast is due to the common lymphopenia caused in patients with COVID-19, which generates a loss of immune tolerance over HB, which itself can cause viral reactivation [126]. However, there is one study showing that COVID-19 was not associated with reactivation or seroconversion in chronic HB patients, despite using immunomodulatory treatment in a short course for severe COVID-19[127].

The current data are controversial and contradictory; therefore, it is necessary to take into account the number of patients and the heterogeneity of the population studied based on HB activity, the presence of cirrhosis, and stage of the liver disease. A recent systematic review and meta-analysis concluded that the association of co-infection of HB and SARS-CoV-2 does not have a serious adverse impact in patients hospitalized with COVID-19[128].

In regards to the treatment of COVID-19 in patients with chronic HB, we must be cautious as the use of corticosteroids or tocilizumab may reactivate HB[129]; although, as previously mentioned, this has not been shown to happen[127]. Finally, the evolution of patients with COVID-19 and HB superinfection is not clear, as the studies have yielded contradictory results and prospective studies with large numbers of patients and control of variables such as presence of other comorbidities, viral replication, cirrhosis, and stage of the liver disease are required. In the case of patients with COVID-19 and recently diagnosed HC, the HC treatment should be postponed until the remission of COVID-19; however, if treatment has already been ongoing, it is necessary to monitor the interactions of HC and COVID-19 treatments[99].

Drug-induced damage

Since the onset of the COVID-19 pandemic, multiple medications have been used as potential treatments, including antimalarials, antiparasitics, antivirals, monoclonal antibodies, *etc*. Some of these medications have hepatotoxic effects, which can be reviewed on the website http://www.livertox.nih.gov, where updated data of all drugs are available (Table 2)[58,99,130]. Of similar relevance is the consideration of drug interactions, as some treatments are experimental. Interactions can be reviewed at: https://www.covid19-druginteractions.org of Liverpool University.

Most of the data collected have come from case reports, particularly of serious cases and cohorts, for which there may be uncontrolled variables, with patients having pre-existing liver disease, interaction with unreported medicines, and use of traditional medicine, herbal products, or substances. Heightened awareness of both hepatotoxicity and drug interactions in patients with COVID-19 must continue, as should further research efforts regarding these interactions.

Table 2 Therapeutic management of patients with coronavirus disease 2019 and hepatotoxicity

Medication	Hepatotoxicity	Action mechanism	Currently recommended use for COVID-19
Hydroxychloroquine	Likelihood score: D (possible). Rare cause of clinically-apparent liver injury	Altered metabolism of other medications	Not recommended
Azithromycin	Likelihood score: A (well-known). Transient and asymptomatic elevation in serum aminotransferases; Typical cholestatic hepatitis	Unknown	Not recommended
Ivermectin	Likelihood score: D (possible). Mild elevation of serum aminotransferases; Reports of acute liver failure	Unknown	Not recommended
Dexamethasone	Likelihood score: A (well-known). Long-term use effects; Symptoms usually represent the worsening or triggering of an underlying liver disease	Drug-associated fatty liver disease	Recommended as emergency use
Remdesivir	Likelihood score: D (possible). Mild to moderate transient elevation of serum aminotransferases	Inhibition of mitochondrial RNA polymerase or idiosyncratic injury	Recommended as emergency use
Lopinavir/ritonavir	Likelihood score: D (possible). Moderate to severe elevation of serum aminotransferases (pattern hepatocellular to cholestatic or mixed); Duration 1-2 mo; Reports of acute liver failure; Caution in patients with co-infection by hepatitis B virus-hepatitis C virus-human immunodeficiency virus	Inhibits both of the isoforms of CYP3A del P450, which may result in production of a toxic intermediate	Not recommended
Baricitinib	Likelihood score: E (unlikely). Moderate transient elevation of serum aminotransferases (17% of patients); Hepatitis B reactivation	Unknown	Recommended as emergency use
Tocilizumab	Likelihood score: C (probably). Mild to moderate transient elevation of serum aminotransferases; Duration 8 wk	Unknown	Recommended as emergency use

COVID-19: Coronavirus disease 2019.

Vaccination controversies

Chronic hepatic disease (CHD) is considered a state of immunosuppression due to a multifactorial state of systemic immunological diffusion[131], which predisposes to infections and a cause of decompensation and mortality in cirrhotic patients[132]. Immunization is, therefore, recommended in patients with cirrhosis and pre-transplantation and post-transplanted patients, with specifications for the different types of vaccines[133]. Inactivated vaccines (*e.g.*, influenza, pneumococcal, viral hepatitis A, viral HB, diphtheria, tetanus, poliomyelitis, and acellular pertussis) are preferred over live attenuated vaccines (*e.g.*, tuberculosis vaccine, measles, mumps, rubella, varicella zoster virus, and Herpes zoster)[132,133].

The development of the SARS-CoV-2 vaccine has evolved favorably with phase 3 trials, offering effectiveness and safety. Currently, 53 vaccines have been authorized by the United States' Federal Drug Administration, including those from Pfizer/BioNTech, Moderna, Oxford/AstraZeneca/Sputnik V, and Janssen. Due to the haste of the trials, very strict inclusion and exclusion criteria have been applied to avoid adverse effects. Patients with CHD are preferably not included. In the Pfizer vaccination study, 217 (0.6%) of 37706 participants had liver disease, and only three (< 0.1%) had moderate to severe liver disease. In the Moderna trial, 196 (0.6%) of 30351 participants had liver disease; the Oxford/AstraZeneca, Sputnik V, and Janssen trials completely excluded patients with pre-existent CHD. On the other hand, trials of the 53 vaccines excluded patients with systemic immunosuppression which involves post-transplant liver patients and AIH patients[114,134,135].

As mentioned, CHD patients are a susceptible and high-risk population for COVID-19 complications, and should be classified as a vulnerable population. It is paramount to define the effectiveness and safety of immunization against SARS-CoV-2. As the development and trial of new vaccines occur and vaccination programs are started, information will be generated in different subgroups of populations, including patients with hepatic disease.

FUTURE RESEARCH DIRECTIONS

After a year of pandemic, the information that has emerged regarding SARS-CoV-2

infection and liver injury in patients without or with pre-existing liver disease has opened the course of new lines of research that should be addressed in future studies. The pathophysiology of COVID-19-induced liver injury is complex and more research is necessary to determine the degree of relevance of each of the described mechanisms. Abnormal liver biochemical parameters have been associated with a more serious course and a worse prognosis in patients with SARS-CoV-2 infection, so the usefulness of such measurements in the identification and staging of those patients with related alterations should be evaluated in depth in prospective studies. It is necessary to investigate the impact of SARS-CoV-2 infection in the clinical course of pre-existing liver disease (*e.g.*, fatty liver disease, viral or AIH, and cirrhosis). Long-term follow-up in liver transplant patients suffering from COVID-19 should be investigated to determine if the infection alters graft viability. It is necessary to include patients with liver diseases in the vaccination protocols, to determine the related effectiveness and safety.

CONCLUSION

Liver injury in patients with infection due to SARS-CoV-2 is a frequent extrapulmonary manifestation, particularly in hospitalized patients, and its presence has been associated with an increased risk of complications, including death. The pathophysiology of liver damage in COVID-19 patients is multifactorial and various mechanisms interact. On the other hand, SARS-CoV-2 infection in patients with pre-existing liver disease (*i.e.*, fatty liver disease, cirrhosis, autoimmune or viral hepatitis, and liver transplant patients) presents an increased risk of an ominous course of the disease. Therefore, the presence of liver damage (both acute onset or as a pre-existing condition) requires close monitoring and individualized management according to the individual conditions of the patients. Further research is required to have a better understanding of the SARS-CoV-2 and liver interaction that can improve the therapeutic approach for patients.

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OPINION REVIEW

Chronic hepatitis B infection with concomitant hepatic steatosis: Current evidence and opinion

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Abstract

With the increasing incidence of obesity and metabolic syndrome worldwide, concomitant nonalcoholic fatty liver disease (NAFLD) in patients with chronic hepatitis B (CHB) has become highly prevalent. The risk of dual etiologies, outcome, and mechanism of CHB with concomitant NAFLD have not been fully characterized. In this review, we assessed the overlapping prevalence of metabolic disorders and CHB, assessed the risk of advanced fibrosis/hepatocellular carcinoma in CHB patients concomitant with NAFLD, and discussed the remaining clinical issues to be addressed in the outcome of such patients. We also explored the possible roles of hepatitis B virus in the development of steatosis and discussed difficultiesof histological evaluation. For CHB patients, it is important to address concomitant NAFLD through lifestyle management and disease screening to achieve better prognoses. The assessment of progressive changes and novel therapies for CHB patients concomitant with NAFLD deserve further research.

Key Words: Nonalcoholic fatty liver disease; Hepatitis B; Metabolic disorders; Steatosis; Mechanism; Disease burden

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Core Tip: The pathophysiology of concomitant hepatitis B and hepatic steatosis remains unclear. This review comprehensively discusses the epidemiology, risk factors, long-term outcomes, histological assessment, potential mechanisms, and therapeutic options in this field. We believe further studies can clarify the interactions of hepatitis B virus and steatosis, and provide novel strategies for the management of hepatitis B patients

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INTRODUCTION

Chronic hepatitis B (CHB) has become highly prevalent worldwide in recent decades, affecting 350 million people, especially in Africa, Latin America and the Asia-Pacific region[1]. Although the incidence of hepatitis B virus (HBV) infection has recently decreased because of the widespread use of vaccines, the number of existing CHB patients remains significant[2]. CHB patients are at risk of severe liver-related adverse events, including decompensation, hepatocellular carcinoma (HCC), and even death. The persistence of covalently closed circular DNA (cccDNA) and incomplete immune tolerance lead to continuing HBV reproduction, resulting in chronic liver inflammation and fibrosis[3]. Despite the availability of potent antiviral treatments, we have not yet been able to eradicate HBV.

Nonalcoholic fatty liver disease (NAFLD) has become epidemic in those with chronic liver disease, with a worldwide annual incidence ranging from 6% to 35%[4]. The constantly increasing prevalence of NAFLD is paralleled by global increases of obesity and insulin resistance[5]. The natural course of NAFLD is asymptomatic and slowly progressive. A considerable proportion of CHB patients have concomitant hepatic steatosis or even steatohepatitis. A number of studies have investigated the relationship between CHB and NAFLD. Current evidence suggests that hepatic steatosis may have a protective effect on CHB by decreasing HBV viral markers, but CHB patients with concomitant NAFLD are faced with increased risks of advanced liver disease and HCC[6]. The management of such patients is challenging. We know little about the mechanisms of the interactions between HBV and steatosis. Therefore, this review was performed to determine the impact of HBV on hepatic steatosis and its underlying mechanisms.

EPIDEMIOLOGY OF STEATOSIS IN CHB

Prevalence and incidence of steatosis in patients with CHB

NAFLD is defined as the presence of steatosis (*i.e.* more than 5% liver fat content) without coexisting etiologies of secondary steatosissuch as alcohol abuse, metabolic dysfunction, and drug-induced liver injury[7]. Of the viral etiologies, hepatitis C virus (HCV) infection is known to influencechanges in insulin resistance and lipid metabolismthat would lead to hepatic steatosis and more severe inflammation in patients with chronic hepatitis C (CHC)[8]. The prevalence of fatty liver in CHC patients has been reported to range from 40% to 80%[9], depending on metabolic status, alcohol abuse and, virus genotypes[10]. Unlike HCV, there is currently no direct evidence that HBV increases the risk of steatosis. Even so, concomitant hepatic steatosis is not uncommon in HBV-infected patients.

NAFLD is reported to account for nearly 25% of the causes of elevated serum alanine aminotransferase (ALT) among CHB persons[11]. The prevalence of biopsy-proven NAFLD in CHB patients has been estimated to range from 14% to 30%[12-17]. Our recent study reported a prevalence of hepatic steatosis in CHB of 17.3%[18].A meta-analysis reported a higher prevalence of 29.6%[19]. Another recent study found a lower prevalence of NAFLD in CHB patients than in controls (13.5% *vs* 28.3%) using proton magnetic resonance spectroscopy, a highly reliable steatosis assay[20]. We performed a meta-analysis that found a lower prevalence of steatosis in CHB than in the general population (Supplementary material). The results of nine studies indicated a negative association with a possible risk for steatosis in CHB (pooled odds ratio (OR)= 0.81, 95%CI: 0.71-0.920, P = 0.001; Figure 1). Furthermore, the incidence of steatosis in a Korean cohort study was significantly lower in CHB patients than in the

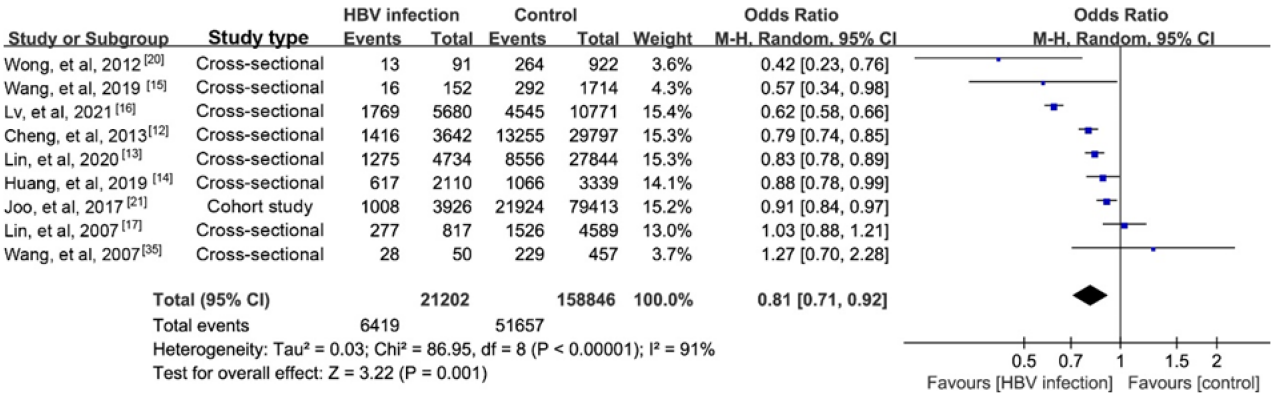


Figure 1 Meta-analysis of the prevalence of hepatic steatosis in patients with hepatitis B virus infection*vs* control.

controls (40.6 *vs* 43.5 per 1000 person-years)[21], and that was lower than an estimate of 52.34 per 1000 person-years in the general population reported by another meta-analysis[22].

Various factors may have contributed to the low prevalence of steatosis in patients with CHB. A study with propensity score analyses reported that a concurrent HBV infection was associated a lower risk of NAFLD than that in subjects who were only hepatitis B core antibody (anti-HBc) positive[23]. Other viral factors, including HBV genotypes, serum HBV DNA level, and hepatitis B e-antigen (HBeAg)positivity, were reported not to be associated with the prevalence of steatosis[20]. Our previous study reported that subclinical hypothyroidism had a role the development of steatosis in CHB patients, and that elevated thyroid stimulating hormone levels, even at normal ranges, were associated with an increased odds ratio of steatosis (OR = 1.54)[24]. Host metabolism has a role the development of steatosis. It was reported that overweight (OR = 5.99), hypertriglyceridemia (OR = 2.95), and type 2 diabetes (OR = 1.88) were risk factors for hepatic steatosis in CHB patients[25,26]. CHB patients with NAFLD presented with altered metabolic profiles and unhealthy lifestyle habits[27,28]. We speculate that differences in the estimated prevalence of NAFLD reported in these studies may be partly explainedby the modified metabolic status in CHB.

Metabolic dysfunctions in CHB

Metabolic dysfunctions have been considered as key factors for incident steatosis in CHB,with oxidative stress, insulin resistance, and hyperglycemia as contributors to hepatic steatosis. Insulin resistance increases fatty acid synthesis, delivery of free fatty acids to the liver, and accumulation of triglycerides in hepatocytes. Chronic inflammatory processes are activated in obesity, type 2 diabetes mellitus (T2DM),and other insulin-resistant states. In this context, activated macrophages release tumor necrosis factor-α and interleukin-6, which promote low-grade inflammation of adipose tissue and even the progression of hepatic damage[29,30]. Proinflammatory cytokines play a crucial role in liver inflammatory responses by promoting hepatocyte apoptosis, hepatic stellate cell proliferation, and angiogenesis[31]. In chronic liver disease, inflammation, fibrosis, and liver function decompensation disrupt liver synthesis functions. Decreased lipoprotein biosynthesis results in lower serum triglyceride and cholesterol levels[32]. Several large studies have described the associations between HBsAg positivity and disorders of lipid metabolism. HBsAg-positive patients had decreased serum cholesterol and triglyceride levels and a decreased prevalence of hyperlipemia [27,33,34]. Our study revealed a lower levels of hepatoxic lipids in serum from NAFLD-HBV patients than in those with only NAFLD[35]. The natural course of HBV infection may play a role in changes in lipid metabolism, especially in elderly patients [36]. This inverse relationship between HBV infection and serum lipid profile may also contribute to reducing the prevalence of metabolic syndrome[37,38].

Another aspect of steatosis in CHB is that impaired glucose and lipid metabolism make intrahepatic lipid content more sensitive to changes in energy intake. Liver inflammation and elevated ALT have been reported to be related to insulin resistance [39,40]. Evidence suggests that the prevalence of insulin resistance is higher in patients with CHB concomitant with NAFLD than in patients with HBV or NAFLD alone[41]. Numerous studies have reported a negative association of CHB and steatosis without aparallel risk associated with insulin resistance[40]. First, decreased liver functional reserve was found to promote insulin resistance, and because it is involved in glucose

metabolism, liver damage from hepatitis causeddisorders of glucose metabolism. The risk of developing diabetes was decreased in CHB after excluding patients with cirrhosis. Second, the association of insulin resistance and steatosis was attenuated by multiple host factors other than viruses, andage and obesity were both confounders of the risk of diabetes in CHB patients[42].

In CHB patients, steatosis resultsfrom a combination of metabolic abnormalities and the status of HBV infection. Thataccounts for the reported differences in the prevalence of steatosis in CHB patients and explains why previous HBV infection does not affect the prevalence of NAFLD[23]. The design of early studies failed to comprehensively evaluate metabolic status, calorie intake, and physical activity of CHB patients. Therefore, it was not possible to adjust for all confounding factors. Causes associated with those factors deserve investigation.

PROGRESSION AND OUTCOMES OF CHB WITH NAFLD

Disease severity of CHB with NAFLD

Chronic HBV infectionand NAFLD are the leading causes of chronic liver disease worldwide. Previous studies have considered steatosis to be an irrelevant or even a protective factor of CHB[25,43],but few focused on the effect of HBV on the severity and long-term outcome of NAFLD. The meta-analysis mentioned above revealed a strong negative association between serum viral load (*e.g.*, HBV DNA level and HBsAg positivity) and hepatic steatosis[19]. Similarly, a Korean cohort with non-CHB controls found an association between HBsAg positivity and a reduced risk of NAFLD [21]. After adjusting for metabolic factors, including insulin resistance, the association was attenuated[21], which indicated that metabolic and viral factors should both be taken into consideration.

Nonalcoholic steatohepatitis (NASH) is a severe form of NAFLD, that is prevalent in CHB patients. In a North American and European cohort, the prevalence of biopsy-proven NASH was approximately 17%[44]. NASH is characterized by necroinflammation and hepatocyte ballooning and is the major cause of advanced liver fibrosis, cirrhosis, and HCC in NAFLD[45]. Compared with bland steatosis, NASH has a more rapid progression in fibrosis[46], and it has been associated with anincreasedincidence of HCC, of up to 5.29 per 1000 person-years[47]. There is no doubt that CHB patients with NASH have a higher risk of developing advanced fibrosis, HCC,or even death than patients without steatohepatitis[44,48]. Concomitant NASH should thus be taken seriously in CHB patients. Hepatic inflammationis key fordisease progression. Although it would be difficult to differentiate the cause of inflammation from steatohepatitis in CHB patients, the risk of disease progression would be decreased if HBV replication could be suppressed before age 40. Therefore, the outcome of CHB patients with NASH would be improved in patients with early-stage NAFLD and low HBV replication phase. Comprehensive assessment and close monitoring are required in the management of CHB patients, irrespective of their viral load.

Risk of fibrosis in CHB patients with NAFLD

In patients with NAFLD, fibrosis is the characteristic that is most closely related to long-term adverse events compared with other histological features[49]. In the development of fibrosis in NASH, sustained lipotoxicity and endoplasmic reticulum stress induce cell death in steatotic hepatocytes. Developmental pathways including Notch, Hedgehog and YAP–TAZ are persistently activated to cope with the chronic insult. As a result, crosstalk of hepatocytes-macrophages-hepatic stellate cells and activation of resident Kupffer cells lead to inflammatory and fibrogenic responses[50].

Accumulating evidence suggests an increased risk of advanced fibrosis and long-term adverse prognosis in CHB patients with NAFLD. Our cross-sectional study found that CHB patients with steatosis had less severe fibrosis than those without steatosis [51]; but in prospective cohort studies, the baseline severity of steatosis was associated with more progressive fibrosis[52-54]. Furthermore, Charatcharoenwitthaya *et al*[25] reported that steatohepatitis but not simple steatosis was an independent predictor of significant, advanced fibrosis.The additive effect of steatosis has also been reported in the progression of fibrosis. Persistent severe steatosis led to a 2-fold increased risk of fibrosis progression over a 3-year follow-up[43]. A retrospective cohort study with biopsy-confirmed cirrhosis progression found that CHB patients with concomitant steatosishad a higher proportion of incident cirrhosis (36%) than those without steatosis (22%)[55]. There is little direct evidence of the effect of steatosis on fibrosis regression. It has been reported that low body mass index (BMI) and steatosis

resolution during tenofovir antiviral treatment were associated with fibrosis regression in CHB patients[43,56], suggesting that management of metabolic disorders and concomitant steatosis were key considerations of anti-fibrotic treatment.

Risk of HCC in CHB patients with NAFLD

Previously, more than 70% of HCC morbidity was attributed to chronic viral hepatitis. NAFLD has been predicted to replace viral etiologies in contributing to the HCC burden. NAFLD could account for more than 30% of HCC cases, especially in developed countries[57]. The progression of HCC in CHB patients with NAFLD is complicated, with direct evidence remaining elusive. As previously discussed, liver fibrosis and cirrhosis are recognized as key drivers of HCC[47]. Evidence suggests that metabolic factors are also responsible for disease progression. CHB patients with high BMI values were reported to have increased incidences of cirrhosis and HCC[58],and long-term follow-up has indicatedthat the incidence of HCC and the risks of liver-related mortality increase with the number of associated metabolic factors[59]. Two retrospective liver biopsy-proven cohort studies reported a 2-7-fold increase in the risks of HCC in CHB patients with NAFLD[48,55]. Recent studies reported similar results, but they found the association was reduced after adjusting for metabolic factors and age[6,60]. We speculate that metabolic factors, especially T2DM, play an important role in the development of HCC.

HCC remains the second leading cause of death related to malignancy worldwide [61].Screening and management of metabolic disorders in CHB patients are crucial for the prevention of HCC, andcoexisting factors should be taken into consideration. In the above-mentioned study, the association of hepatic steatosis and HCC development was observed only in patients receiving antiviral treatment, not in the overall population. That is because confounding factors including significant alcohol drinking were not considered[48]. In addition, the prevalence of NAFLD andthe HBsAg seroclearance rate both increase with age[62]. Therefore, patient age may be a confounding factor in the association of HBV infection with the long-term outcome of NAFLD. Noninvasive methods have often been used to identify steatosis in population-based studies, considering the injury risk of liver biopsy and the infeasibility of large numbers of patients,and using different measurements leads to bias in the definition of steatosis. Trial-based studies have carefully selected homogeneous patient samples that were matched for the presence of confounders. If patients with significant metabolic dysfunctions such asT2DM and cardiovascular disease were excluded, then the study results might not be representative of all types of real-world situations.

The overall long-term outcome of patients with CHB concomitant with steatosis is subject to avariety of risk factors. Liver conditionsincluding NASH and advanced fibrosis were found to have additive effects on event-free survival (HCC, decompensation and transplantation)[44]. Wong *et al*[52] reported that steatosis had no direct predictive effect on these events including cardiovascular events, liver-related complications, malignancy and mortality.

Important issues in clinical management

The effects of steatosis on the progression and remission of CHB have been widely investigated but few studies have focused on the outcome of NAFLD in the natural course of CHB or during antiviral treatment. Issues that should be addressed are: (1) The incidence of NAFLD in CHB and decreased risk of NAFLD in CHB[21] and diabetes[26]. Metabolic factors including weight change and lifestyle habits have not been comprehensively evaluated but a negative association may not reflect the etiology; (2) The progression of fibrosis in NAFLD needs study because the findings of cross-sectional studies are inconsistent. Concurrent HBV infection has been associated with advanced fibrosis[63], but anti-HBc-positive NAFLD patients are reported to have increased risks of cirrhosis, HCC, and liver-related complications[64]. The role of HBV infection status requires investigation; (3) The regression of fibrosis in NAFLD needs study. Steatosis resolution has been reported to be associated with fibrosis regression in CHB[43]; but whether HBV cures or antiviral treatment responses affect fibrosis regression in NAFLD remains unknown; and (4) The resolution of NASH. Given the interaction of steatosis resolution and fibrosis regression, the impact of fibrosis improvement after antiviral treatment of steatosis-related inflammation remains unknown.To address these issues, the interaction between HBV and metabolic homeostasis in the progression of liver disease requires further study.

EFFECT OF ANTIVIRAL TREATMENT ON NAFLD

Few studies have investigated the incidence of hepatic steatosis during antiviral treatment with pegylated interferon and nucleos(t)ide analogs (NAs). NA therapy reduces HBV replication, suppresses inflammation, and improves fibrosis in CHB[56]. Most studies have shown that NAFLD has no impact on viral suppression and biochemical responses during NAs antiviral treatment[65,66]. Whereas, decreased virological responses were also observed in CHB patients concomitant with steatosis in several studies[43,67,68]. In those cases, the authors speculated that the elevated ALT caused by NAFLD could lead to premature antiviral treatment and a poor response.

A recent study reported that lamivudine, entecavir, or adefovir dipivoxil increased the BMI and increased the visceral fat area in CHB patients[69]. It is worth noting that tenofovir disoproxil fumarate (TDF) and tenofovir alafenamide (TAF) were found toimprove the lipid metabolic profile of CHB patients. Compared with patients treated with entecavir, greater declines in serum lipid components were observed in patients treated with TDF[70]. An *in vitro* study reported that TDF modulated lipid metabolism by upregulating hepatic CD36 by activating PPAR-α[71]. Overexpression of hepatic CD36 improved hepatic steatosis and insulin resistance by reducing hepatic lipids, which might explain the findings above. In a study of CHB patients, switching to TAF improved metabolic dysfunction, reduced serum ALT levels, and improved ALT normalization in patients with or without diabetes despite significant increases in body weight and BMI[72].

Myrcludex B is a novel agent for CHB treatment that inhibits hepatic bile acid uptake transporter Na^+ taurocholate cotransporting polypeptide (NTCP). It has been shown to be safe and welltolerated and is currently in phase 2b clinical trials for the treatment of HBV infection. Recently, a study showed that Myrcludex B induced weight loss and decreased hepatic adiposity by inhibiting the hepatic clearance of bile acids from portal and systemic blood, stimulating glucagon-like peptide-1 (GLP-1) secretion[73].Because these agents potentially improve dyslipidemia and metabolic dysfunctions, TDF, TAF and Myrcludex B could be used to treat metabolic diseases, including NAFLD. They may be the best choice for CHB patients with concomitant NAFLD.

MECHANISMS OF INTERACTION BETWEEN HEPATITIS B AND STEATOSIS

Currently, the majority of CHB patients are on antiviral treatments that provide potent virological suppression. Viral factors are attenuated, and the relative influence of metabolic factors are increased in the course of NAFLD[74], which was verified in a study in HBsAg transgenic (HBs-Tg) mice. High-fat methionine-choline-deficient diet (MCD)-fed HBs-Tg mice had more liver fat accumulation and macrovesicular fat droplets than wild-type C57BL/6 mice. HBsAg increased susceptibility to steatohepatitis in those mice[75]. The evidence indicates that CHB patients should manage their lifestyle to prevent the incidence of NASH.

Accumulating evidence on single nucleotide polymorphisms (SNPs) and NAFLD severity and progression has helped to elucidate the genetic basis of NAFLD. SNPs of patatin-like phospholipase domain-containing protein 3 (PNPLA3) and transmembrane 6 superfamily member 2 (TM6SF2) are two common genetic determinants of NAFLD[76,77]. In cohort studies of biopsy-proven CHB patients, several SNPs of PNPLA3 were independently associated with steatosis, lobular inflammation, and steatohepatitis andwere similar to the findings of NAFLD studies[48,78,79], in which patients with SNPs of the T allele of rs1010023 in PNPLA3 were more susceptible to hepatic steatosis[78]. The T allele of rs58542926 in TM6SF2 has been associated with altered lipids and hepatic steatosis in CHB patients; this substitution was associated with increased HBV DNA[80]. As the T allele has a low prevalence of 7% worldwide, it may play a role in steatosis in a minority of the population. The evidence suggests the possibilityof genetic susceptibility to fatty liver in CHB.

Clinical studies that describe macroscopic results are often limited by the heterogenous characteristics of enrolled patients. Basic science studies would better balance confounding factors, and provide clues for elucidating the mechanism of interactions between HBV infection and fatty liver. Hepatitis B protein X (HBx), one of the four HBV proteins, has an important role in HBV infection. Previous studies in HepG2-HBx stable cells and in HBx-transgenic mice confirmed that overexpression of HBx induces

hepatic lipid accumulation, and that HBx is a risk factor for steatosis[15]. HBx is mediated by sterol regulatory element binding protein 1 (SREBP-1) and peroxisome proliferator-activated receptor gamma (PPAR-γ)[81]. HBx has been reported to upregulate fatty acid binding protein 1 (FABP1) to promote hepatic lipid accumulation in the development of steatosis in HBV-induced cells[82]. During treatment, the expression of HBx and downstream factors were downregulated by antiviral agents [83], which might be helpful for the improvement of steatosis during antiviral treatments in clinical studies.

As a regulator of adipocyte differentiation, CCAAT/enhancer-binding protein α (C/EBPα) triggers adipocyte differentiation by inducing complex cascades of transcription. In HBx-transfected hepatocytes, HBx stimulates the expression and transcriptional activation of C/EBPα and PPAR-γ[81]. Endoplasmic reticulum stress is associated with liver injury and fibrosis. C/EBPα is also the effector of endoplasmic reticulum stress, but whether HBV-induced endoplasmic reticulum stress plays a role in the development of concomitant steatosis requires further research. The involvement of adiponectin in adipogenic conversion in CHB has been extensively studied. Adiponectin improves hepatic insulin sensitivity and decreases lipid accumulation in macrophages. CHB patients have been reported to have higher serum adiponectin levels[27,84], which could account for the low prevalence of steatosis in HBV-infected subjects. The metabolic changes related to HBV infection at the cellular level could help explain the clinical,but phenotypic differences related to NAFLD at the individual level require further study.

CHALLENGES IN HISTOLOGICAL EVALUATION

NAFLD and CHB use different scoring systems for histological assessment. The fatty liver inhibition of progression algorithm and steatosis, activity, and fibrosis (FLIP-SAF) score[85] and NAFLD activity score (NAS)[86] are used to evaluate histological activity.The criteria include steatosis, lobular inflammation, hepatocyte ballooning, and fibrosis. In the assessment of CHB, Ishaket al[87] and The METAVIR study group [88]have described two major scoring systems to evaluate necroinflammation and fibrosis. Because the pathogeneses of CHB and NAFLD are complex, the coexistence of HBV and steatosis-induced injury may affect each other. The steatosis distribution patterns in CHB patients with concomitant NAFLD and in those with NAFLD alone. In CHB, SHG/TPEF scores of the steatosis distribution and in the peripheral region and that in lobule region were similar.In NAFLD, the steatosis percentage was significantly lower in the peripheral region than in the lobule region[89]. Whether CHB concomitant with NAFLD has novel pathophysiological characteristics remains unclear.

The inflammation of CHB and NAFLD has been differentially evaluated by hepatocyte injury. The modified Knodell necroinflammatory score of the Ishak scoring system is used to assess CHB activity and is based on four variables, periportal or periseptal interface hepatitis, confluent necrosis, focal apoptosis and portal inflammation[87]. The NAS and SAF activity scoresare used to quantify inflammation in NAFLD. Ballooning is the most specific inflammatory characteristic of NAFLD, andin CHB concomitant with NAFLD, ballooning is predictive for clinical outcomes[44]. A cross-sectional studyreported that CHB with steatosis had less necroinflammation and fibrosis than CHB without steatosis[19], but CHB activity has not been associated with the degree of steatosis[90]. Although both algorithms score fibrosis on a scale of from 0 to 4, they are based on different zones and severities. In contrast to viral hepatitis, fibrosis characteristic of NASH is predominantly seen with lobular inflammation. Thus, zone-3 perisinusoidal fibrosis has been the primary focus during evaluations [86].

The dynamic assessment of inflammation and fibrosis are major problems faced in evaluating CHB concomitant with NAFLD. During antiviral treatment, viral suppression attenuates necroinflammation in CHB, inducing fibrosis improvement. Although the pathogenesis of HBV infection and NASH differ, they share a common pathway to fibrogenesis because of necroinflammation. Histological improvement in CHB is defined as a more than 2-point reduction in the Knodell necroinflammatory score with no worsening of fibrosis.Resolution of NASH is defined an inflammation score of 0 to 1 and a ballooning score of 0[91,92]. It is difficult to determine whether changes in CHB inflammation severity influence the NAFLD inflammation score orwhether fibrosis regression in CHB induces improvement of NAFLD. These problems have complicated the assessment of CHB regressionconcomitant with

NAFLD.Currently, with the new nomenclature of metabolic-associated fatty liver disease[93], it is no longer a diagnosis of exclusion. Based on the presence of steatosis and metabolic dysfunction, the diagnosis of NAFLD coexisting with CHB might be more feasible[94]. Thus, new definitions are needed to correctly classify patients during histopathological evaluation in clinical practice.

CONCLUSION

The decreased prevalence and incidence of steatosis in CHB patients are mainly due to altered metabolic profiles. However, concomitant steatosis increases the occurrence of adverse liver-related events, including cirrhosis and HCC. Lifestyle management and screening of metabolic changes associated with steatosis are recommended in CHB patients regardless of viral load. Traditional antiviral therapy has no impact on the incidence of steatosis, but tenofovir and NTCP inhibitors have strong metabolic effects, which could be promising in the treatment of CHB patients concomitant with NAFLD. Further study is necessary to determine whether these associations cause macro changes. As the mechanisms of interactions between steatosis and HBV infection become more clear, future studies will provide novel strategies for the clinical management and treatment of CHB concomitant with NAFLD.

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REVIEW

Acute kidney injury and hepatorenal syndrome in cirrhosis

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Abstract

Acute kidney injury (AKI) in cirrhosis, including hepatorenal syndrome (HRS), is a common and serious complication in cirrhotic patients, leading to significant morbidity and mortality. AKI is separated into two categories, non-HRS AKI and HRS-AKI. The most recent definition and diagnostic criteria of AKI in cirrhosis and HRS have helped diagnose and prognosticate the disease. The patho-physiology behind non-HRS-AKI and HRS is more complicated than once theorized and involves more processes than just splanchnic vasodilation. The common biomarkers clinicians use to assess kidney injury have significant limitations in cirrhosis patients; novel biomarkers being studied have shown promise but require further studies in clinical settings and animal models. The overall management of non-HRS AKI and HRS-AKI requires a systematic approach. Although pharmacological treatments have shown mortality benefit, the ideal HRS treatment option is liver transplantation with or without simultaneous kidney transplantation. Further research is required to optimize pharmacologic and nonpharmacologic approaches to treatment. This article reviews the current guidelines and recommendations of AKI in cirrhosis.

Key Words: Acute kidney injury; Hepatorenal syndrome; Liver cirrhosis; Treatment; Biomarkers; Prognosis

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cirrhosis as well as hepatorenal syndrome. We review the most current topics including diagnosis, current definitions, pathophysiology, novel biomarkers, treatment, pharmacology, nonpharmacologic treatment, and topics of further research.

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INTRODUCTION

Acute kidney injury (AKI) is a relative decrease in a kidney's glomerular kidney function (GFR) and frequently occurs in patients. The incidence of AKI ranges from 20%-50% in cirrhotic patients when hospitalized for acute decompensation[1-6]. AKI imparts significant morbidity and mortality in patients with liver cirrhosis. Hospitalized cirrhotic patients have a high mortality rate, both inpatient and post-discharge [7]. Cirrhosis itself is a complex disease process that causes significant morbidity due to substantial volume shifts and increased vasodilation. Renal dysfunction, therefore, imparts another layer of complexity to those with cirrhosis and must be considered when a patient is being evaluated for liver transplantation (LT)[8].

Renal function is a weighted parameter in the Model for End-Stage Liver disease (MELD) score[9,10]. By accounting for creatinine, the MELD score allows patients with renal failure (acute or chronic) to receive liver transplants promptly[9,10]. Renal disease is an increasing health care burden in the United States as there has been a rise in the prevalence and incidence of type II DM and obesity along with chronic liver disease. Rustgi *et al*[11] calculated the additional cost of chronic kidney disease (CKD) in chronic liver disease patients by stage[11].

In the 1960s, Hecker and Sherlock described the process of renal dysfunction with the presence of ascites in advanced cirrhosis and defined it as hepatorenal syndrome (HRS)[12,13]. HRS is renal dysfunction resulting from systemic hemodynamic effects of portal hypertension secondary to liver cirrhosis[12], AKI in liver cirrhosis has been separated into non-HRS-AKI and HRS. The latter has been subdivided into type 1 HRS, known more recently as HRS-AKI, or type 2 HRS, known as HRS-CKD. The current recommendations and literature involving AKI and HRS in patients with liver cirrhosis are reviewed here.

DIAGNOSIS (NON-HRS-AKI)

The definition of HRS relies first and foremost on the definition of AKI. The definition of AKI has evolved. The first challenge has been determining the most accurate and available renal function measurement, which is the calculation of GFR. There is, however, no consensus on the most accurate method to measure GFR. Traditionally, the definition of AKI has been based on urine output and serum creatinine (sCr). The diagnosis of AKI is dependent on the patient's baseline sCr. The International Club of Ascites (ICA) defines a baseline sCr as the last sCr within three months of current sCr [14].

The definition of AKI historically has gone through many updates as enumerated in Table 1[14-17]: Given the complexity of cirrhosis, AKI in cirrhosis needed its definition with specific criteria. In 2004, AKI was defined by the Acute Dialysis Quality Initiative (ADQI) group using the RIFLE criteria and divided into the three stages (stage 1 or R, stage 2 or I, or stage 3 or F)[15]. Further updates by the AKI Network (AKIN) and Kidney Disease Improving Global Outcomes (KDIGO), which labeled the stages 1-3 [14-17]. Numerous consensus definitions have defined AKI. KDIGO is the most recent consensus definition for AKI that was updated in 2012[17]. In 2010, the ADQI with the ICA defined criteria for AKI in liver cirrhosis as shown in Table 2[18-20].

The guidelines were again updated in 2015 by the ICA to adopt the 2012 KDIGO definition of AKI. The benefit of the KDIGO criteria over the AKIN criteria for AKI is removing the absolute creatinine value of at least 1.5 mg/dL as a requirement, sCr in

Table 1 A brief overview of the consensus definitions of acute kidney injury

Criteria	Stage	Definition
RIFLE criteria/ADQI in 2004[15]		At least 1.5 × baseline serum creatinine within 7 d, decrease in urine output of 0.5 mL/kg/h for 6 h, decrease in GFR of at least 25%
	Stage 1 (R)	1.5 × baseline Cr, GFR decrease of 25%, UOP < 0.5 mL/kg/h for 6-12 h.
	Stage 2 (I)	2 × baseline serum creatinine, decrease of GFR < 50%, UOP < 0.5 mL/kg/h for 12 h
	Stage 3 (F)	3 × baseline serum creatinine, decrease of GFR of 75%, UOP < 0.3 mL/kg/h for 24 h, anuria for 12 h, or on RRT acutely
Acute Kidney Injury Network (AKIN) in 2007 [16]		Definition: increase of at least 0.3 mg/dL in last 48 h, 1.5 × baseline creatinine in last 48 h, or UOP < 0.5 mL/kg/h for at least 6 h
	Stage 1	Increase of 0.3 mg/dL w/in 2 d, 1.5-2 × baseline serum creatinine within 2 d, or UOP < 0.5 mL/kg/h for 6-12 h
	Stage 2	2-3 × baseline serum Cr, UOP < 0.5 mL/kg/h for at least 12 h
	Stage 3	3 × baseline serum Cr, UOP < 0.3 mL/kg/h for 24 h, anuria for 12 h, on RRT
Kidney Disease Improving Global Outcomes (KDIGO) in 2012[17]		Increase in sCr of at least 0.3 mg/dL within 48 h, increase of at least 1.5 × baseline in the last 7 d, or urine output < 0.5 mL/kg/h for at least 6 h
	Stage 1	Increase of 0.3 mg/dL, 1.5-2 × baseline Cr, UOP < 0.5 mL/kg/h for 6-12 h
	Stage 2	2-3 × baseline serum Cr or UOP < 0.5 mL/kg/h for at least 12 h
	Stage 3	3 × baseline serum Cr, increase of 0.5 mg/dL above absolute level of 4.0 mg/dL, on RRT, UOP < 0.3 mL/kg/h for 24 h, or 12 h of anuria

GFR: Glomerular kidney function; UOP: Urine output; RRT: Renal replacement therapy; ADQI: Acute Dialysis Quality Initiative; Cr: Creatinine.

Table 2 The current and past consensus definitions of acute kidney injury in cirrhosis

Criteria	Stage	Definition
ADQI/ICA in 2010[19]		The absolute increase in serum Cr of at least 0.3 mg/dL or 1.5 × baseline serum creatinine
	Stage 1	Increase of 0.3 mg/dL within 48 h or 1.5-2 × baseline serum creatinine
	Stage 2	Increase of 2-3 × baseline serum Cr
	Stage 3	At least 3 × baseline serum Cr with an increase of 0.5 mg/dL or currently on RRT
ICA-AKI in 2015[14]		An absolute increase in serum Cr of at least 0.3 mg/dL within 48 h or 1.5 × baseline Cr level within the last 7 d
	Stage 1A	Increase of 0.3 mg/dL from baseline in 48 h, 1.5-2 × baseline serum creatine. Absolute value of serum Cr < 1.5 mg/dL
	Stage 1B	Increase of 0.3 mg/dL from baseline in 48 h, 1.5-2 × baseline serum creatine. Absolute value of serum Cr > 1.5 mg/dL
	Stage 2	Increase of 2-3 × baseline
	Stage 3	Greater than 3 × baseline Cr, Cr > 4 mg/dL with rise of > 0.5, or on RRT

RRT: Renal replacement therapy; ADQI: Acute Dialysis Quality Initiative; ICA: International Club of Ascites; AKI: Acute kidney injury; Cr: Creatinine.

patients with cirrhosis may underestimate renal dysfunction due to low baseline muscle mass[14]. However, in staging AKI, as stressed by Angeli *et al*[14], the absolute level of 1.5 mg/dL was used to differentiate between stage 1-A and stage 1-B[14], as shown in Table 2. The new ICA criteria emphasize the importance of having a baseline sCr for making the diagnosis and allow for a prior sCr within three months to be considered a baseline[14].

DIAGNOSIS (HRS)

HRS is defined as renal dysfunction in chronic liver disease (usually severe or advanced cirrhosis) or acute liver failure[1,8,14]. HRS has primarily considered a

diagnosis of exclusion with specific criteria explained in Table 3, and its two types are generally differentiated by disease course. However, it may be challenging to differentiate from acute tubular necrosis (ATN). Table 3 lists the definitions of HRS types 1 and 2[21]. Type 1 and 2 HRS were renamed HRS-AKI and HRS-CKD in 2015. The most significant difference between the prior diagnosis of HRS type 1 and HRS-AKI has been eliminating an absolute sCr level of 2.5 mg/dL[21-23].

PATHOPHYSIOLOGY OF HRS

HRS has been theorized to be caused by various mechanisms. The most well-understood hypothesis evokes splanchnic vasodilation changes, leading to increased peripheral vasoconstriction[24,25]. Additionally, there is evidence for other processes. Hepatocytes and stellate cells are known to produce vasodilatory mediators, including nitric oxide, prostacyclin, carbon monoxide, endogenous cannabinoids, adrenomedullin[1,8,26-28]. The destruction of hepatocytes leads to an increased release of these products into the splanchnic circulation, resulting in significant arterial vasodilation. This, in turn, decreases the systemic mean arterial pressure, causing compensatory activation of the sympathetic nervous system resulting in the consistent release of norepinephrine, angiotensin II and antidiuretic hormone[8,26-31]. These processes trigger unopposed vasoconstriction in the renal arteries *via* multiple physiologic mechanisms to counteract the splanchnic vasodilation and preserve renal function. As cirrhosis progresses, the systemic vascular resistance is decreased to the point that an increase in cardiac output cannot compensate adequately to maintain adequate organ perfusion[8,25] (Figure 1). This phenomenon is described as cirrhotic cardiomyopathy, directly related to sustained portal hypertension[1,32,33]. The possibility of spontaneous bacterial peritonitis (SBP) must be accounted for every time a patient is treated for AKI[2,34,35].

PATHOPHYSIOLOGY OF NON-HRS AKI

The typical forms of non-HRS-AKI include prerenal azotemia (PRA), parenchymal renal disease, and drug-induced kidney injury. Prerenal AKI accounts for up to 60% of all AKI cases in patients with cirrhosis[2,34]. The most common causes of AKI in cirrhosis are hypovolemia, SBP, bacterial infections (other than SBP), sepsis, upper gastrointestinal bleeding, and shock. Infections and sepsis (urinary tract infections, pneumonia, skin infections, or SBP) cause decreased blood flow to the renal vasculature and cause kidney injury for cirrhosis patients who are already susceptible to volume shifts[3-5,36,37]. Frequent large-volume paracentesis can cause hypovolemia, exacerbated by increased third spacing and hemodynamic instability[7]. Gastrointestinal bleeding also causes hypovolemia and is commonly implicated in renal dysfunction[3-5,36,37]. Common drugs which can contribute to AKI in cirrhosis are diuretics and laxatives, particularly lactulose. Intrinsic renal dysfunction is present in around 30% of AKI cases in cirrhosis[34,35]. Intrinsic renal disease plays a role in AKI as well. Many of the insults that affect liver function and are common etiologies in cirrhosis can lead to acute and chronic kidney disease. These can include autoimmune disease, medications, hepatitis B infection, and hepatitis C infection[7].

There are cirrhosis-specific mechanisms that also contribute to non-HRS AKI. Hepatic inflammation has been well-described in the literature for contributing to non-HRS AKI[12,38]. In the setting of cirrhosis or chronic liver disease, inflammation may be the result of damage-associated molecular patterns (DAMPs) in hepatocytes and gut immunity weakening from pathogen-associated molecular patterns (PAMPs)[12,39]. DAMPs specific to the liver include interleukin (IL)-1, IL-33, and bile acids recognized by the Kupfer cells' toll-like receptors[12,40]. Gut bacterial translocation has been associated with the release of PAMPs (*e.g.*, lipopolysaccharide), or DAMPs (*e.g.*, heat shock proteins), from a cirrhotic liver leading to a systemic inflammatory response which can lead to the development of non-HRS AKI[12,41-45] (Figure 1).

Adrenal insufficiency is also frequently present in patients with cirrhosis. A retrospective study by Moini *et al*[46] evaluated 105 cirrhotic patients and reported that 15% of cirrhotic patients had some degree of adrenal insufficiency and identified hyponatremia and elevated international normalized ratio as risk factors for its development[46,47]. These processes can decrease glucocorticoids' synthesis and result in adrenal insufficiency[48]. Inadequate adrenal response subsequently alters cardiovascular hemodynamics through vascular tone changes and cardiac output

Table 3 The previous and current definition and nomenclature of hepatorenal syndrome[14,19,21-23]

	Previous and current definition and nomenclature
Criteria to confirm of HRS *vs* other etiology of renal dysfunction	To diagnose HRS, patients must have: (1) The presence of ascites; (2) No improvement of creatinine after holding diuretics; (3) No improvement after 48 h of albumin supplementation (1 g/kg/d); (4) No signs of shock; (5) No recent nephrotoxic medications (antibiotics, contrast, NSAIDs); and (6) No signs of kidney disease (proteinuria, microhematuria, no findings on renal ultrasound)
HRS type 1 (most recent definition in 2007)	Rapid renal injury (within two weeks) defined by 2 × baseline serum creatinine to a value > 2.5 mg/dL or 50% reduction in creatinine clearance
HRS type 2	Moderate renal failure with creatinine ranging from 1.5 to 2.5 mg/dL that occurs progressively
Definition of HRS-AKI	Patients with the criteria above and ICA-AKI 2015 definition for AKI
Definition of HRS-CKD	Patients who meet the criteria in row 1 and the rise of serum creatinine and changes in urine output are all progressive (> 1 wk)
	Patients with HRS-CKD are known to have decreased urine output over weeks to months

ICA: International Club of Ascites; AKI: Acute kidney injury; HRS: Hepatorenal syndrome; CKD: Chronic kidney disease; NSAID: Non-steroidal anti-inflammatory drug.

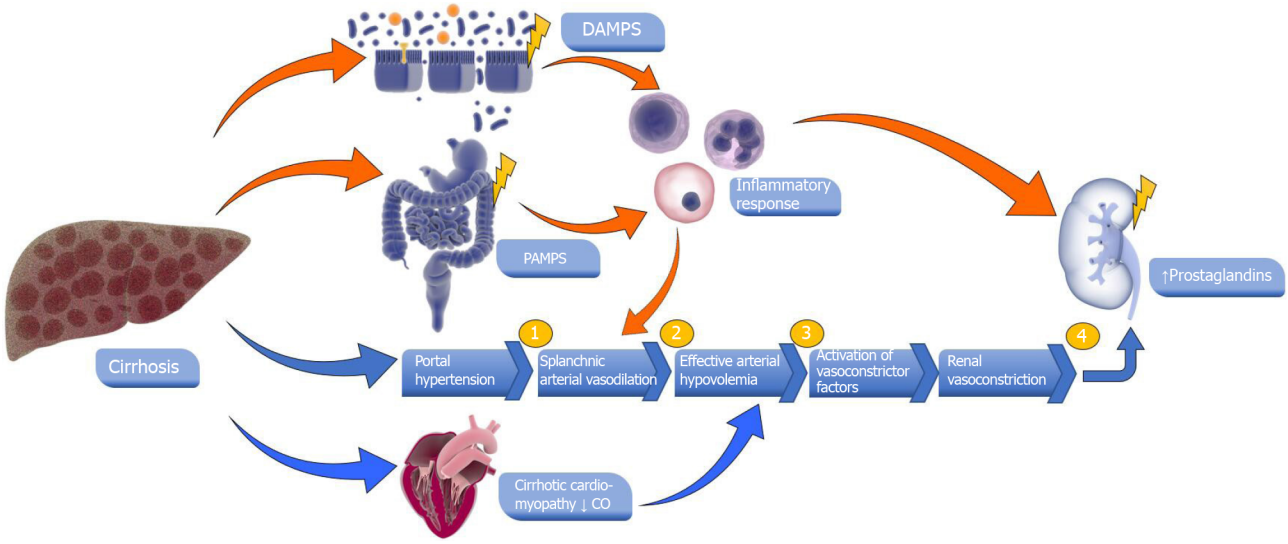


Figure 1 Pathogenesis of hepatorenal syndrome and acute kidney injury in cirrhosis. (1) Patients with cirrhosis present with a marked splanchnic arterial vasodilation due to portal hypertension; (2) Splanchnic vasodilation causes a decrease in systemic vascular resistance leading to effective arterial hypovolemia; (3) There is activation of endogenous vasoconstrictors such as the renin-angiotensin-aldosterone system, sympathetic nervous system and arginine vasopressin; and (4) The activation of these systems leads to renal vasoconstriction inducing a decrease in glomerular filtration rate and development of hepatorenal syndrome. A decrease in cardiac output may contribute to a decrease in effective arterial blood volume. Pathogen-associated molecular patterns and damage-associated molecular patterns, derived from bacterial translocation and from injured liver, may activate circulating innate immune cells, leading to an inflammatory response. The Inflammatory mediators may lead to impairment of circulatory dysfunction and consequently, kidney tissue damage. Library of Science & Medical Illustrations were utilized in part to create this figure (https://creativecommons.org/Licenses/by-nc-sa/4.0/). DAMPs: Damage-associated molecular patterns; PAMPs: Pathogen-associated molecular patterns.

leading to decreased renal perfusion[46].

In patients with nonalcoholic steatohepatitis (NASH), studies have shown that around 28% have worsened renal function[46,49]. Patients with NASH/nonalcoholic fatty liver disease (NAFLD) and CKD have been shown to alter the renin-angiotensin system[46,50]. In patients with metabolic syndrome and NAFLD, alterations in the renin-angiotensin system with increased renin/angiotensin II receptor activation (from increased activation of angiotensin-converting enzyme-2) have been linked to hepatic steatosis, fibrosis and leading to NASH cirrhosis. This same process is well established to cause physiologic changes in the kidney, such as efferent artery vasoconstriction, which initially causes glomerular hyperfiltration and leads to hypertrophy with eventual scarring[46,50]. Other mechanisms in patients with NASH cirrhosis include 5′AMP-activated protein kinase activation, lipoprotein dysmetabolism, and oxidative damage through downregulation of sirtuin-1[46,51,52]. Patients with NAFLD/NASH will have comorbidities such as hypertension and diabetes mellitus and are highly

susceptible to AKI[34,35].

In viral hepatitis, the most common kidney injury mechanism involves creating immune complexes with the virus, antibodies against infected hepatocytes, or direct cytopathic impact[46,53]. Hepatitis B infection is associated with polyarteritis nodosa (PAN), membranous nephropathy, and membranoproliferative glomerulonephritis[54,55]. Pathologically, renal biopsies generally reveal immune complex deposition, particularly hepatitis B envelope antigen in membranous nephropathy[55]. Chronic hepatitis C infections are also often linked with glomerular disease. The most common renal dysfunction causes include mixed cryoglobulinemia, PAN, and membranous nephropathy[56].

BIOMARKERS

Early recognition of AKI and accurate measurement of renal function in cirrhosis is crucial when treating patients. Still, AKI can often be missed due to the baseline abnormalities present in patients with cirrhosis. Urine output is not an accurate measurement of a patient's renal function or GFR in cirrhosis. Third-spacing causes urine output to drop, which underestimates renal function. At the same time, diuretic use may lead to an overestimation of renal function.

The most frequently used laboratory value to measure GFR is sCr because it is readily available, inexpensive, and accurate[57-60]. However, sCr has many factors that influence its value, such as race, age, gender, and muscle mass[18,60]. In cirrhosis, patients are malnourished, cachectic, and sarcopenic, leading to a deficiency in protein intake and is associated with muscle wasting[61]. These patient-specific factors are why creatinine may be lower in cirrhotic patients leading to an overestimation of GFR and renal function. Another factor leading to inaccuracy in creatinine correlating with GFR is that hyperbilirubinemia affects Jaffe's kinetic assay that measures sCr and leads to an inaccurately low measurement[18,59].

sCr remains the primary measurement of renal function in cirrhosis because the use of novel biomarkers remains experimental[59]. Urinary sodium and the fractional excretion of sodium (FeNa) have only been used as an adjunct to sCr to help diagnose HRS and PRA[23].

NOVEL BIOMARKERS

Given that sCr may not evaluate the degree or the timing of AKI promptly, novel biomarkers with promise are being evaluated[59,62]. Cystatin C is a low-molecular-weight protein that is produced by all nucleated cells. It is filtered by the glomerulus and mainly reabsorbed by the proximal tubule[63]. Cystatin C testing is less readily available and is more expensive. Despite the limitations, cystatin C is not affected by age, muscle mass, malignancy, or inflammation[64,65]. The assay, unlike sCr, is not affected by high levels of serum bilirubin[66]. Prior studies have not had sufficient evidence of superiority for cystatin C in comparison to Cr. However, combination equations of Cr and cystatin C are superior to sCr[64,65]. Cystatin C is an independent predictor of AKI and outcomes, including mortality[67,68]. Other biomarkers of interest include neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), IL-18, and liver-type fatty acid-binding protein (L-FABP)[18,59,69]. The biomarkers' clinical benefits and limitations are described in Table 4.

NGAL is a small protein made by the kidney, lung, stomach, and colon[70,71]. Using mouse and rat models, Mishra *et al*[70] in 2003 demonstrated that NGAL was upregulated in prerenal AKI and ATN setting and that increased urinary NGAL could be detected within 2 h of initial renal injury[70]. Multiple studies have evaluated the efficacy and utility of urinary NGAL in cirrhotic patients with AKI. When urinary NGAL was used to define and predict morbidity in AKI, the authors concluded that urinary NGAL levels were elevated in ATN compared to PRA or HRS-AKI. However, the most significant confounder in its utility is the overlap between ATN's lower values and HRS's upper values or PRA[18,72-75]. Two studies had found that urinary NGAL was superior to cystatin C in utility for diagnosis of AKI or ATN[75,76]. In contrast, Barreto *et al*[74] studied 132 cirrhotic patients hospitalized with infections. The authors found that among patients with persistent AKI, HRS-AKI could be accurately predicted with urinary NGAL values lower than 86 μg/g creatinine in 88% of patients[74]. In a study with 55 patients, Lee *et al*[77] found that urinary NGAL levels were significantly higher in ATN than HRS and PRA. Also, median urinary

Table 4 The most well-known novel biomarkers being studied for acute kidney injury in cirrhosis

Novel biomarker	Source	Benefits/Clinical uses	Limitations
Cystatin C[62-68]	Plasma, urine	Early biomarker of AKI, potential benefit with severity of disease. Unaffected with age, sarcopenia, gender, or sepsis. Unaffected by malignancy and serum bilirubin level. Multiple studies found it to be an independent risk factor of AKI and mortality	Increased levels in CKD. Influenced by low levels of albumin. Potentially influenced by elevated WBC and CRP. Takes longer time to result when compared to sCr
NGAL[18,67-79]	Urine	Found in kidney tubular cell that is released during damage or injury. Elevated in AKI in cirrhosis and potential predictor of mortality. Markedly elevated in ATN, mildly elevated in prerenal azotemia/CKD/HRS-AKI	Increased levels in CKD. Increased levels in infections, particularly urinary tract infections. Overlap with values in PRA, HRS, and other AKI types of AKI. Small quantities are made in the liver
IL-18[75,78,82-84]	Urine	Very similar to urinary NGAL. Markedly elevated in cirrhotic patients with ATN, in comparison to other AKI types. Found in monocytes and macrophages. A notable proinflammatory marker. Not confounded by CKD, sepsis or UTI	There are increased levels in PRA and HRS but significant overlap in values with limited clinical utility. Levels are increased in levels of inflammation in the kidney other than AKI
Kidney Injury Molecule-1[18,73,84-86]	Urine	Originally found in kidney tubular transmembrane protein. Not expressed in normal kidney tissue. Noted with increased levels in ATN in cirrhosis when compared to the other types of AKI in cirrhosis. High specificity for ischemic or nephrotoxic kidney injury	Elevated from inflammatory conditions. Found to have overlap between different forms of AKI. Confounded by presence of infection
L-FABP[87-93]	Urine	Found in kidney proximal tubule. Levels may be increased in AKI or AKI 2/2 sepsis. Potential utility in predictor in adverse outcomes including AKI in patients with chronic liver disease and other liver disease	Limited studies in cirrhosis. Found to be increased in CKD. Increased in acute liver injury and liver failure as well

AKI: Acute kidney injury; HRS: Hepatorenal syndrome; CKD: Chronic kidney disease; ATN: Acute tubular necrosis; UTI: Urinary tract infection; NGAL: Neutrophil gelatinase-associated lipocalin; PRA: Prerenal azotemia; CRP: C-reactive protein; WBC: White blood cell; sCr: Serum creatinine; IL: Interleukin.

NGAL levels in HRS were markedly different from PRA levels, and the authors found that NGAL was an independent risk factor for mortality with AKI[77]. Jaques *et al*[67] studied multiple biomarkers in AKI in 55 decompensated cirrhosis patients. Compared to the non-AKI patients, they found that urinary NGAL levels are higher in ATN than PRA and HRS. However, HRS urinary NGAL levels had an intermediate pattern[67]. Urinary NGAL predicted poor outcomes in patients as well[67]. Kim *et al*[68] studied urinary NGAL and cystatin C in 328 decompensated cirrhosis patients (41 patients with AKI). The authors found that urinary NGAL is a predictor of AKI and outcomes (including mortality)[68]. Recently, Huelin *et al*[78] studied urinary NGAL and IL-18 on 320 cirrhosis patients with AKI. Urinary NGAL was elevated in AKI progression during hospitalization and was predictive of AKI progression in conjunction with MELD score. Urinary NGAL was significantly elevated in ATN when compared to hypovolemia-induced AKI and HRS-AKI[78]. Currently, there are no definitive diagnostic thresholds for differentiation between these types of AKI[79-81]. Urinary NGAL does not have an established role in the diagnosis, prediction, or prognosis of AKI in cirrhosis, but more promising results in extensive studies may change that. Another significant limitation is the expense of the test.

IL-18 is a proinflammatory cytokine expressed in the proximal tubule. It is released in urine when the cells are damaged in AKI[75]. Urinary IL-18 is elevated in patients with AKI, especially from ischemic injury, but urinary IL-18 is not elevated in conditions such as urinary tract infections, nephrotoxic injury, and CKD[75,82,83]. Tsai *et al*[84] in 2013 evaluated the clinical outcomes of 168 cirrhotic patients with AKI and severe sepsis. They found that urinary IL-18 was significantly higher in patients with ATN than patients with functional AKI, proposing a cutoff of 708.5 pg/mg creatinine to differentiate between the two groups. Urinary IL-18 was found to be a stronger predictor of ATN than serum IL-18. However, the authors were unable to conclude if urinary IL-18 could distinguish ATN from HRS-AKI. Clinically, they found that elevated urinary IL-18 was associated with higher hospital mortality[84]. Huelin *et al*[78], a study previously mentioned, studied IL-18 compared to urinary NGAL and found that it had a lower accuracy to predict ATN *vs* other forms of AKI[78].

KIM-1 is elevated in AKI from ischemic injury to the proximal tubule[83,84]. Belcher *et al*[73] evaluated KIM-1 in patients with AKI with other etiologies (PRA, ATN, and HRS) and found that ATN was the most elevated with overlap with HRS[73]. Other studies found that in patients with cirrhosis, elevations in urinary KIM-1 levels were increased mainly in ATN compared to other AKI presentations and could serve as a prognostic indicator[73,85,86].

L-FABP is a small protein found in the proximal tubular epithelium and binds to free fatty acids when reabsorbed in the proximal tubule[87]. L-FABP may be elevated in sepsis and specific etiologies of CKD (diabetic nephropathy or glomerulonephritis) [88]. Yamamoto *et al*[89] studied L-FABP in animal and human models (12 kidney transplant patients) in response to AKI[89]. The authors reported an increase in levels of L-FABP in mice models with prolonged exposure to ischemia to the kidneys, particularly during ischemic reperfusion injury. Doi *et al*[90] evaluated urinary L-FABP in 145 mice and 145 septic shock patients with AKI. L-FABP was high in septic shock patients with AKI and higher in the patients who did not survive[90]. L-FABP has been studied in acute liver failure and chronic liver disease and not just HRS and AKI in cirrhosis[91]. In patients with acetaminophen included acute liver failure, serum L-FABP levels were lower in survivors when compared to patients who passed away [92]. Eguchi *et al*[93] studied L-FABP in 242 chronic liver disease patients (chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma). The authors found that serum L-FABP increased in liver cirrhosis compared to chronic hepatitis and is higher in the presence of hepatocellular carcinoma. L-FABP correlates with kidney function markers, especially BUN, creatinine, and GFR[93]. This study does show the potential for L-FABP in chronic liver disease and other complications, including AKI. Serum L-FABP may have many clinical utilities in acute and chronic liver disease, including AKI; however, more large-scale studies should be performed to ascertain exact clinical utility.

Two new biomarkers being studied for potential benefits are insulin-like growth factor binding protein-7 and tissue matrix metalloproteinase inhibitor-2. However, there is not enough evidence to note potential utility. They are only approved for evaluating AKI in patients with intensive care unit (ICU) and need further evaluation [94]. Novel biomarkers can differentiate both the degree of renal dysfunction and possible etiology, but the data are not substantial enough to currently recommend utility. Additionally, these tests are not readily available and are expensive methods to evaluate renal function.

TREATMENT (INITIAL TREATMENT OF AKI IN CIRRHOSIS)

In AKI injury, clinicians must recognize and intervene as soon as possible. In patients with cirrhosis, all factors possibly contributing to AKI must be recognized promptly [14,20,37,95]. All unnecessary nephrotoxic medications such as Non-steroidal anti-inflammatory drugs should be discontinued and avoided altogether. Beta-blockers for variceal prophylaxis or other comorbidities should be evaluated for risk *vs* benefits[96, 97]. In patients with PRA or dehydration, diuretics should first be discontinued as excessive diuresis is a common cause of kidney dysfunction in cirrhosis patients[20]. Excessive diarrhea from high doses of lactulose is another potential cause[20]. Patients with gastrointestinal bleeding should be transfused if indicated. Patients should have screening for infectious etiology, and patients should be placed on antibiotics immediately along with appropriate volume supplementation if an infection is diagnosed[98-100].

Clinicians should attempt a trial of volume expansion for the patients, but crystalloid, colloid, or blood products are dependent on etiology and clinical judgment. If a patient requires large-volume paracentesis, 6-8 g of albumin *per* liter of fluid removed after 5 L should be administered.

Therapeutic response is defined as improving serum creatine to at least 0.3 mg/dL near the baseline. However, even with adequate improvement, patients should be screened frequently to prevent a recurrence. Recommendations currently include an initial screen 2 to 4 d after discharge with a 2-4 wk follow-up for the first six months after discharge[14,36]. Patients with stage 2 or 3 AKI should be suspected of HRS-AKI, and HRS-AKI management should be initiated. Figure 2 provides a brief algorithm that can be used when first approaching AKI in a cirrhotic patient.

TREATMENT (HRS-PHARMACOTHERAPY)

The patient meets the HRS criteria if there is no creatinine improvement after the withdrawal of all nephrotoxic agents and volume expansion with 1 g/kg/24 h for 48 h [14]. The patient should receive prompt pharmacologic therapy, which entails starting vasoconstrictor therapy with albumin supplementation to avoid cardiac output loss or loss of effective circulating volume[1,101]. The vasoconstrictors utilized for treatment

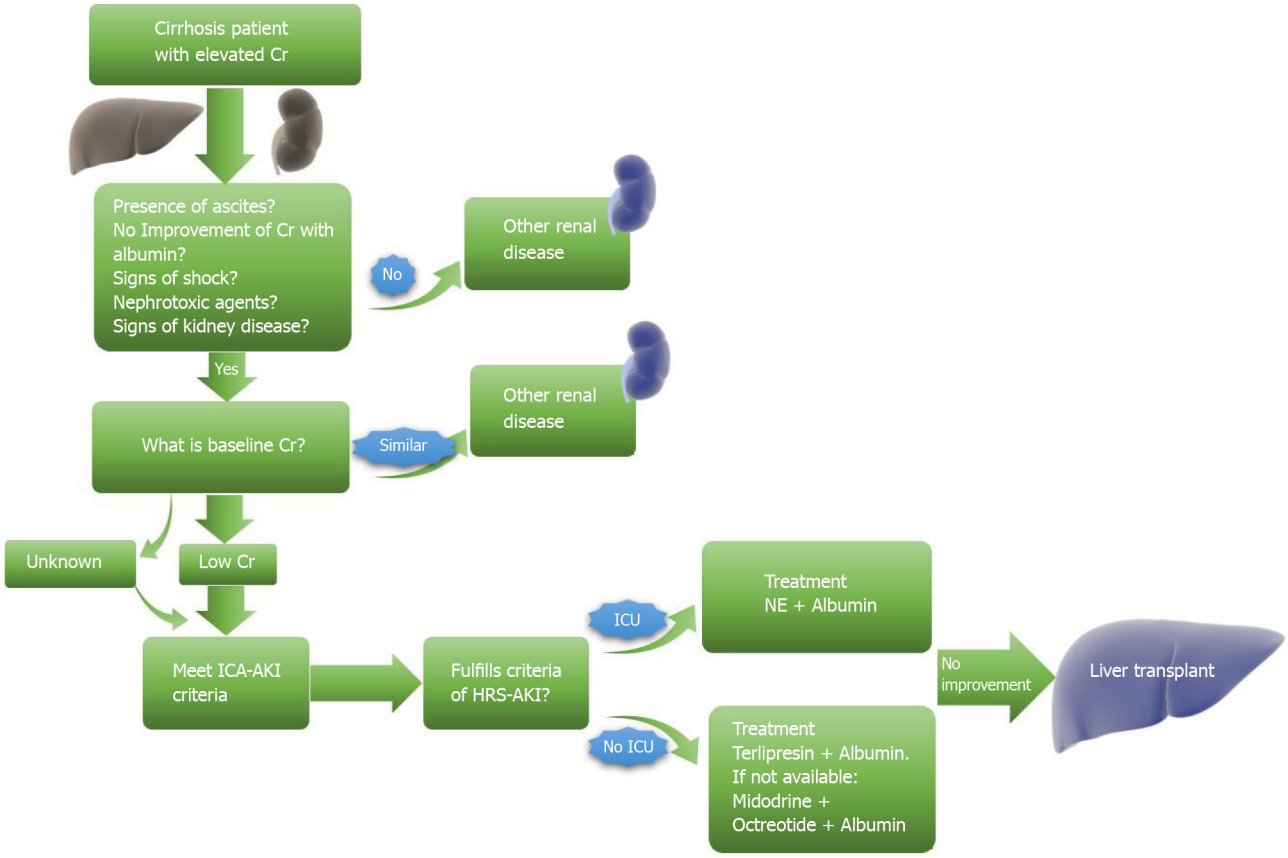


Figure 2 Algorithm of the diagnosis and treatment of hepatorenal syndrome. The algorithm indicates differential diagnosis, diagnosis of hepatorenal syndrome (HRS) and HRS treatment. Library of Science & Medical Illustrations were utilized in part to create this figure (https://creativecommons.org/Licenses/by-nc-sa/4.0/). Cr: Creatinine; ICA: International club ascites; AKI: Acute kidney injury; HRS: Hepatorenal syndrome; ICU: Intensive care unit; NE: Norepinephrine.

are terlipressin, noradrenaline, octreotide, and midodrine[102-106]. The treatment goal is cited to be a goal sCr of 1.5 mg/dL or less with a reduction of at least 50%.

Terlipressin has been the most extensively studied and has the most robust evidence of efficacy in treating HRS-AKI of the three vasoconstrictor therapies with known superiority to octreotide and midodrine[101-108]. Terlipressin is more effective with fewer adverse effects when given in continuous infusions than bolus administration [99-108]. Over the years, multiple trials proved the efficacy of terlipressin with albumin as an effective treatment of HRS type 1[101,103-105,108-113]. A recent phase 3 trial by Wong *et al*[114] studied 300 patients using terlipressin and albumin compared to the placebo group. They found a significant improvement of HRS versal and renal function but was significantly associated with adverse events, including respiratory failure[114]. Serious adverse effects include angina, dysrhythmia, hypertension, and peripheral ischemia (intestines, fingers, scrotum). Patients with ischemic cardiomyopathy or peripheral vascular disease should not be treated with terlipressin[110]. Currently, it is not available in the Unoited States.

Noradrenaline has alpha-adrenergic properties that promote vasoconstriction with fewer effects on contractility[111,115]. Patients treated with noradrenaline require central venous access and require close, frequent monitoring in the ICU[116]. In their prospective study, Gupta *et al*[117] found norepinephrine to be an effective treatment for HRS reversal in 30 patients[117]. Multiple randomized controlled trials (RCTs) have compared noradrenaline to terlipressin[102,111,118-121]. Alessandria *et al*[118], in their pilot unblinded RCT, evaluated 22 patients comparing terlipressin and noradrenaline. The difference in HRS reversal was 83% and 70%, respectively, but there was no mortality difference[118]. Singh *et al*[119], Sharma *et al*[102], and Goyal *et al*[121] evaluated noradrenaline *vs* terlipressin and found them to have comparable efficacy and safety to improve HRS renal function[102,119,121]. Liu *et al*[122], in a randomized, double-blinded trial with 617 patients with septic shock found no significant difference in 28-d mortality between terlipressin compared to noradrenaline[122]. These studies have bolstered the use of noradrenaline, which is less expensive and more readily available in most countries. Consequently, Arora *et al* [123] in an open-label RCT, found that terlipressin, when compared to noradrenaline,

showed significant improvement in the reversal of HRS (40% *vs* 16.7%), day 4 response (26.1% *vs* 11.7%), day 7 response (41.7% *vs* 20%) and in 28-d survival (48.3% *vs* 20%) [123].

The third vasoconstrictor therapy that is commonly used is midodrine in conjunction with albumin and octreotide. Midodrine is an alpha-adrenergic agonist that is frequently used in patients with orthostatic hypotension, and octreotide is a somatostatin analog that physiologically is meant to antagonize the primary pathophysiology of HRS[124,125]. In a pilot study, Angeli *et al*[124] evaluated the efficacy of octreotide, and midodrine found it to reverse HRS in around 40% of the patients with type 1 HRS[124]. It is recommended to utilize the regimen if terlipressin and noradrenaline are contraindicated or unavailable[116]. In 2009, Skagen *et al*[126], in a retrospective study, evaluated the use of octreotide, midodrine, and albumin in 75 patients and found that it improved short-term renal function and survival compared to the group who did not receive them[126].

Many patients, unfortunately, do not respond appropriately to pharmacologic therapy. After 14 d, all medications should be discontinued, and further nonpharmacologic treatment options must be considered.

TREATMENT (HRS-TRANSJUGULAR INTRAHEPATIC PORTOSYSTEMIC SHUNT)

Transjugular intrahepatic portosystemic shunt (TIPS) has been considered for the treatment of HRS, particularly HRS-AKI. Physiologically, treating portal hypertension should improve renal function in HRS; however, in practice, TIPS can cause transient ischemia to the liver, which can lead to acute on chronic liver failure. This may precipitate and worsen renal function in HRS, leading to increased mortality[1]. While several prospective studies have shown a significant benefit in renal function and mortality, they are limited by small size, lack of control groups, selection bias, and strict inclusion/exclusion criteria. The most extensive prospective study compared 31 transplant-ineligible patients with HRS (14 with HRS-AKI and 17 with HRS-NAKI) who underwent TIPS to 10 transplant-ineligible patients who did not undergo TIPS. The 3-mo survival rates were 81% for the group undergoing TIPS and 10% for the TIPS-ineligible group[127]. A 2018 meta-analysis of studies including 128 patients with HRS who underwent TIPS showed pooled 1-year survival rates of 47% in HRS-AKI patients and 64% in HRS-NAKI and renal improvement in 83% of patients[128]. While these results are certainly encouraging, randomized trials with adequate control groups are still lacking. Therefore, TIPS may be appropriate in specific clinical contexts but, at this time, is not routinely recommended in the treatment of HRS.

TREATMENT (HRS-RENAL REPLACEMENT THERAPY)

Renal replacement therapy (RRT) (hemodialysis) is not a treatment for HRS-AKI and is only meant to be a bridge for recovery of liver function or LT. RRT recommendations for cirrhosis patients are the same as for the general population (refractory volume overload, refractory electrolyte imbalance, refractory acidosis, uremia, or intoxication) [116]. Zhang *et al*[129], in a retrospective study, evaluated RRT in patients with HRS type 1 who did not respond to pharmacologic therapy. The study concluded that it did not improve mortality (30-d or 180-d survival)[129]. Patients who are not deemed transplant candidates are not considered candidates for RRT[130].

TREATMENT [HRS-LIVER REPLACEMENT THERAPY (ALBUMIN DIALYSIS)-MOLECULAR ADSORBENT RECYCLING SYSTEM]

A molecular adsorbent recirculating system (MARS) is a form of albumin dialysis which circulates albumin to remove cytokines and bacterial products to combat vasodilation[12]. A 2010 RCT with 189 patients with acute-on-chronic liver failure (50% had HRS AKI) revealed a statistically significant reduction in sCr compared to medical management. However, overall mortality in 28 d was not significantly different in patients with HRS AKI[131]. In 2013, a trial by Lavayssière *et al*[132] studied MARS and found that compared to a control, MARS was able to lower

bilirubin and sCr compared to the control group[132]. However, many studies did not show any significant improvement in creatinine or GFR after MARS. The RELIEF trial failed to show a statistically significant improvement in mortality compared to medical therapy[131]. Due to the equivocal results of all the trials evaluating MARS, the European Association for the Study of the Liver (EASL) does not recommend MARS for HRS treatment but suggested a further investigation into its potential benefits.

TREATMENT [HRS-LIVER REPLACEMENT THERAPY (ALBUMIN DIALYSIS)-BIOARTIFICIAL LIVER SUPPORT SYSTEMS]

Another approach studied to bridge patients with cirrhosis to transplant or recovery includes bioartificial liver support systems. Several types exist, but all generally involve integrating animal or human hepatocytes into a bioreactor to filter toxins. These technologies continue to be studied in both clinical and preclinical trials, showing some promise in acute liver failure[133]. However, large-controlled trials are needed to understand better their role in the treatment of AKI in patients with acute on chronic liver failure.

TREATMENT (HRS-PREVENTION)

Multiple studies have evaluated possible mechanisms to prevent HRS in patients from common causes. When treating infections in cirrhotic patients, there is evidence that albumin administration may have a protective role against HRS. The current recommendation to prevent HRS in SBP is albumin administration at a dosage of 1.5 g *per* kg on day 1 and 1 g *per* kg on day 3[134,135]. This albumin administration regimen has been found to reduce the incidence of HRS and overall mortality in SBP[134,136]. However, these results have not been replicated in other infections[136-138]. An RCT by Guevara *et al*[137] reported that renal function and circulatory function were significantly improved in the treatment group compared to the control with fewer cases of HRS type 1[137]. Another RCT by Thévenot *et al*[138] reported that albumin therapy delayed renal failure, but the 3-mo renal failure rate was not significantly improved. The authors cautioned using large amounts of albumin in critically ill cirrhotic patients[138]. SBP prophylaxis with norfloxacin has been studied and found to lower HRS incidence and improve survival[136,139].

TREATMENT (HRS-TRANSPLANTATION)

The only definitive treatment of HRS refractory to pharmacologic therapy is LT. The use of creatinine in the MELD score has demonstrated the increased importance for patients with renal dysfunction (HRS-AKI or HRS-CKD) to undergo LT. In the setting of HRS, Boyer *et al*[140] reported a survival advantage of 100% *vs* 34% in patients with HRS treated with terlipressin and LT compared to patients treated with terlipressin alone[140]. Although LT remains the only definitive treatment of HRS-AKI, the role of the liver and even simultaneous liver-kidney transplant (SLK) remains unclear in the setting of non-HRS-AKI. In a large retrospective study comparing survival in HRS-AKI patients after undergoing SLK *vs* cirrhotic patients with non-HRS-AKI undergoing the same, HRS-AKI patients' survival post-transplant was significantly superior to those in the non-HRS-AKI group[141].

The percentage of liver transplant recipients undergoing SLKs has substantially increased over the last 18 years. The increase in SLK is likely partly due to the adoption of the MELD score by the Unified Network for Organ Sharing in 2002. The MELD score places significant weight on sCr and imparts a high and increasingly higher transplant priority to progressive renal dysfunction patients. Guidelines for SLK, developed in 2012, were modified in 2017. For patients with cirrhosis and CKD, SLK was recommended for patients with epidermal GFR (eGFR) less than 60 mL/min for at least 90 d before listing or eGFR less than 35 mL/min during the time of listing or inherited metabolic disease[142]. In patients with cirrhosis and AKI, there must be a combination of dialysis and eGFR < 25 mL/min for six weeks[143].

PROGNOSIS

AKI in cirrhosis has a high mortality rate, with 26% of patients dying before discharge [7]. Multiple studies show that the disease course and prognosis of AKI in cirrhosis depend on numerous factors-etiology of kidney injury, multiorgan dysfunction, stage of AKI upon diagnosis and progression of AKI, and lack of response to treatment[7]. Jenq *et al*[144], using the RIFLE criteria, found mortality of 134 cirrhotic patients admitted to the ICU to be 32.1% without AKI, 68.8% with RIFLE-R, 71.4% with RIFLE-I, and 94.8% with RIFLE-F[144]. However, the results were not reliable as patients admitted to the ICU usually have multiorgan dysfunction. The AKI stage directly correlates with in-hospital mortality and post-transplant mortality. Wong *et al*[145] found that the 30-d mortality of patients who do not recover from AKI was 80% *vs* 15% for those who recover[145]. Huelin *et al*[146] in a cohort of 547 patients, found a 90-d transplant-free survival to be 84% with stage 1A AKI, 58% with stage 1B AKI, 48% with stage 2 AKI, and 43% with stage 3 AKI compared to 89% with patients without AKI[1,146]. Bucsics *et al*[147], in a 239-patient retrospective study in 2015, also found that the 30-d mortality increased with increased stage of AKI on diagnosis or progression[147]. Mortality with AKI is markedly increased with complications of cirrhosis, including hepatic encephalopathy and ascites. In a retrospective study, Mindikoglu *et al*[148] reviewed 6917 cirrhotic patients between 2004 to 2014 who developed AKI during hospitalization and were subsequently discharged, and the authors calculated a 32% 90-d mortality and 48% 1-year mortality with higher rates in patients with pre-existing renal disease[148]. Although their study population was primarily male, this was one of the very few studies that studied post-discharge outcomes for patients, as most studies involved inpatient mortality only. Makar *et al* [149] studied the National Inpatient Sample data of 2016 and concluded that of the 6733 hospitalized cirrhosis patients who had AKI that patients with AKI had increased risk of mortality (OR: 8.09; 95%CI: 6.68-9.79; $P < 0.0001$) and prolonged hospital stay by 3.68 d (95%CI: 3.42-3.93; $P < 0.0001$)[149]. Another study found that community-acquired AKI had increased morbidity (progression to CKD) and mortality rates compared to hospital-acquired AKI[150]. In 2020, Tariq *et al*[151], in a meta-analysis of 18747 patients with cirrhosis (from 30 selected studies), found an in-hospital morality up to 6-fold higher in patients with AKI. Important risk factors were noted to be MELD score, Child-Pugh Turcotte stage C, presence of ascites, and sepsis (with or without shock)[151].

Once HRS of either type is diagnosed, it imparts a grave prognosis with median survival for HRS-AKI and HRS-NAKI determined to be about 1 and 6.7 mo, respectively[152]. Importantly, in all the studies evaluating AKI mortality in cirrhosis, the two types of AKI with the highest mortality were AKI-HRS and ATN[4,6,146,153]. Piano *et al*[6] also studied hospitalized patients with cirrhosis and ascites and AKI using the AKIN stage and found that patients who met the ICA criteria for HRS-AKI had the highest mortality[6]. Fagundes *et al*[4] found that patients with HRS or infection-related AKI had the highest mortality[4].

CONCLUSION

Regardless of type, AKI remains a severe complication to cirrhosis patients and a significant challenge for physicians tasked with treating it. Its incidence has increased as definitions shift to recognize and account for the unique clinical and laboratory abnormalities present in cirrhosis. Differentiating HRS-AKI from non-HRS-AKI is essential as the treatments vary, and early interventions may improve outcomes. Transplantation continues to be the only definitive therapy for HRS-AKI as more data are needed to support the use of less invasive strategies such as TIPS and liver replacement therapy. As our understanding of these diseases' pathophysiology and progression evolve, novel biomarkers and directed therapies will hopefully evolve as well.

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REVIEW

Progress and challenges in the comprehensive management of chronic viral hepatitis: Key ways to achieve the elimination

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Abstract

Chronic viral hepatitis is a significant health problem throughout the world, which already represents high annual mortality. By 2040, chronic viral hepatitis due to virus B and virus C and their complications cirrhosis and hepatocellular carcinoma will be more deadly than malaria, vitellogenesis-inhibiting hormone, and tuberculosis altogether. In this review, we analyze the global impact of chronic viral hepatitis with a focus on the most vulnerable groups, the goals set by the World Health Organization for the year 2030, and the key points to achieve them, such as timely access to antiviral treatment of direct-acting antiviral, which represents the key to achieving hepatitis C virus elimination. Likewise, we review the strategies to prevent transmission and achieve control of hepatitis B virus. Finally, we address the impact that the coronavirus disease 2019 pandemic has had on implementing elimination strategies and the advantages of implementing telemedicine programs.

Key Words: Hepatitis C; Hepatitis B; Vaccination; Elimination program; Telemedicine; Direct antiviral agents

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threat to public health by 2030. Despite notable advances reached to achieve those goals, many challenges persist, such as guarantee access to complete vaccination schemes for hepatitis B virus and universal screening for all adults at least once in life to screen for hepatitis C virus. Those non-vaccinated against hepatitis B virus guarantee access to effective therapies programs to all patients who need it, emphasizing risk groups like prison inmates, sex workers, injecting drug users, and men who have sex with men, trying to reduce the high incidence of viral hepatitis in these groups. Telemedicine and telementoring approaches are valuable strategies to facilitate more patients access to healthcare systems and should be encouraged. Coronavirus disease 2019 pandemic affects all strategies significantly to eliminate viral hepatitis, particularly in low-income and middle-income countries. With available effective vaccines for anti-severe acute respiratory syndrome-coronavirus-2, strategies to immunize most people are crucial to restarting the viral hepatitis elimination programs throughout the world as soon as possible.

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INTRODUCTION

More than 320 million people worldwide have chronic viral hepatitis. Around 248 million people are living with hepatitis B virus (HBV) chronic infection, which represents 3.2% of the global population[1-3]; and an estimated 71 to 80 million individuals (1.1%) are living with hepatitis C virus (HCV) chronic infection[3,4].

Chronic viral hepatitis and its related complications, cirrhosis and hepatocellular carcinoma (HCC), have been regarded as the leading causes of death for decades[1], causing globally more than 1 million deaths each year[5]. In fact, by 2040, deaths from chronic viral hepatitis are expected to exceed the related mortality as a whole from human immunodeficiency virus infection (HIV), tuberculosis, and malaria[5,6]. Liver disease due to viral hepatitis represents a substantial burden in the Asia-Pacific region. This region lives 1.8 billion people, which means around 25% of the world's population; a third of global deaths occur due to viral hepatitis, mainly driven by cirrhosis and HCC. Asia-Pacific represents 40% of the global burden of chronic hepatitis, where 115 million people in the Western Pacific are chronically infected with HBV and 14 million with HCV. Al least 58.6% of deaths due to cirrhosis and HCC in the Asia-Pacific region are related to HBV or HCV[7]. In 2013, China was the country that reported the most significant absolute number of deaths and disability-adjusted life-years attributable to viral hepatitis[1].

The North of Africa and the Middle East are also geographic regions extensively affected by viral hepatitis. They have a wide range of viral hepatitis causes, viremic prevalence, and diversity in HBV and HCV genotype distributions. Vaccination and treatment policies, socioeconomic conditions, and migration are responsible factors for the high prevalence of viral hepatitis in these particular regions. Here, elimination strategies might be challenging to implement because of a scarcity of reliable and profitable quality epidemiological data on hepatitis[8].

SEARCH METHODS

It is a narrative review. We searched PubMed, EMBASE, MEDLINE, and Web of Science from January 2015 to January 2021 to identify all studies documenting achievements and challenges on vaccination, diagnosis, access to healthcare systems, therapy, and elimination programs on hepatitis B and hepatitis C viral infections. The following search terms alone or matched with the Boolean operators "AND" or "OR" were used: "Hepatitis C," "hepatitis B," "World Health Organization (WHO)'s goals," "vaccination," "detection," "access to diagnosis," "access to healthcare system," "direct

antiviral agents," "sofosbuvir-velpatasvir (SOF-VEL)," "glecaprevir-pibrentasvir (G-P)," "entecavir (ETV)," "tenofovir disoproxil fumarate (TDF)," "tenofovir alafenamide (TAF)," "elimination program," "telemedicine," "coronavirus disease 2019 (COVID-19)". Using these terms, we found a total of 13497 articles; no study design or language restrictions were applied. We focused on full-text articles, but abstracts were considered if relevant. Finally, we selected the those with the most relevant content.

WHO GOALS FOR 2030

WHO goals are to achieve a 65% reduction in liver-related deaths, which means preventing more than 7 million related deaths by 2030, achieving a 90% reduction in viral hepatitis incidence, and reaching 90% of patients living with viral hepatitis diagnosed by 2030[9-12]. Specifically, in the case of HCV infection, the reduction in liver-related deaths is today achievable since the disponibility of direct-acting antivirals (DAAs), which have a high rate of sustained viral response (SVR). Nevertheless, an increase in harm reduction programs and treatment among populations at risk of transmission is undoubtedly still needed to reduce new infections[9].

For HBV infection, the WHO aims are divided into two main categories: First, prevention of new HBV cases through vaccination and blood safety; second, identification, linkage to care, and treatment of persons living with HBV who need it[10].

THE EFFECTIVE AND SAFE CURE FOR HEPATITIS C

In the absence of an effective vaccine, the cornerstone to achieving HCV elimination worldwide is treatment with DAAs[2], which have excellent efficacy and good tolerability profiles, offering a unique opportunity[13,14]. Currently, pan-genotypic regimens are available, which allows them to simplify decisions when initiating HCV therapy and ensuring universal access for these patients[15].

SOF-VEL is a pan-genotypic regimen that allows achieving the SVR in more than 95%. It can be prescribed even in decompensated cirrhosis because SOF-VEL is a protease inhibitor-free regimen proven effective and safe in this clinical scenario; HCV-infected liver posttransplant recipients are also effectively and safely treated with it SOF-VEL[16-29]. Several cohort studies also have validated the efficacy and safety of SOF-VEL in the real world[30-33]. Despite nearly 80% of SOF being renally excreted[4], the treatment with SOF-VEL is safe. It can be prescribed, achieving a SVR rate greater than 95% in patients with hepatitis C and end-stage renal disease, even in those requiring dialysis[34].

G-P, also a pan-genotypic regimen, is effective and safe in those without cirrhosis and with compensated cirrhosis[35-50] but is contraindicated in decompensated cirrhosis since glecaprevir is a protease inhibitor[4,15]. G-P is effective and safe in patients with end-stage renal disease[51-53]. The study MAGELLAN-2 validated that G-P is a safe and effective therapy to treat HCV infection in those patients who received a liver or kidney transplant[54].

Both pan-genotypic regimens, SOF-VEL, and G-P are also effective and safe in patients coinfected with HIV[55-57].

Around 5% of patients with chronic HCV infection treated with the first line DAAs do not achieve SVR; for this group of patients, sofosbuvir-velpatasvir-voxilaprevir (SOF-VEL-VOX) for 12 wk is the current option of rescue[4,15]. In a study including 137 patients who failed a previous combination of DAAs, a SVR of 95% was reached with SOF-VEL-VOX. Factors related to the reduced rate of SVR were genotype 3 and cirrhosis[58]. Even in those coinfected HIV-HCV patients who failed a previous combination of DAAs, the RESOLVE study demonstrated that 12 wk of SOF-VEL-VOX was safe and effective. The treatment response was not diminished by HIV coinfection [59].

Sixteen weeks of G-P treatment is an effective and safe option for those who failed NS5A or NS3-protease inhibitors[50,60,61]. In a randomized study including genotype 1 patients who failed previous treatment with SOF plus an NS5A inhibitor, retreatment with G-P achieved the SVR in greater than 90% of cases, including patients with compensated cirrhosis[60].

DAAS AND THE LIVER TRANSPLANT PROGRAMS

Since DAAs represent a highly effective and safe therapy, livers from HCV-infected donors can now be used to transplant, optimizing the transplant opportunity for more patients. After transplantation from an HCV-positive donor, the occurrence of HCV infection in HCV-negative recipients is practically universal, requiring post-transplant antiviral treatment[62].

Some interesting strategies are being studied to reduce HCV infection likelihood in organ recipients from HCV-infected donors. Feld *et al*[62] found that ezetimibe (10 mg; an HCV entry inhibitor) plus G-P (300 mg/120 mg) given previous and during 7 d after transplant avoided the occurrence of chronic hepatitis C in 30 (100%) recipients of different organs from HCV-positive donors.

Although patients with HCV infection had a higher risk of post-liver transplant (LT) graft failure and death in the pre-DAA era, this issue seems to be solved in the post-DAA era[63]. The burden of HCV-related LT waitlist and LT is declining in the DAA era, with improved post-transplant outcomes[64]. It probably reflects the impact of DAAs on bettering post-LT results in patients with hepatitis C and maybe also a better patient selection for a LT after 2014[63]. After the availability of DAAs, HCV as an indication for LT has reduced, patients exhibit a less severe disease at transplantation, and there is a trend towards better patient survival[65,66].

Overall listing rates for decompensated HCV cirrhosis have decreased in the DAA era. According to Bittermann and Reddy[67], waitlist recovery is more frequent for HCV patients post-DAAs [adjusted survival hazard ratio 1.78 *vs* pre-DAAs, 95% confidence interval (95%CI): 1.58-2.02; $P < 0.001$], while improvements in waitlist mortality by era are similar to non-HCV candidates [adjusted survival hazard ratio 0.74 (95%CI: 0.7-0.78; $P < 0.001$) and 0.77 (95%CI: 0.74-0.8; $P < 0.001$), respectively][67].

THE STRATEGIES TO CONTROL HEPATITIS B TRANSMISSION AND TO CONTROL THE BURDEN OF DISEASE

Universal vaccination is the essential strategy to prevent HBV transmission. Already in 1992, WHO recommended introducing universal childhood vaccination all around the world. Nowadays, at least 180 countries have adopted this recommendation[68]. The efficacy of universal vaccination programs has been demonstrated in several countries all around the world. In Taiwan, the prevalence of hepatitis B surface antigen (HBsAg) decreased notably from 14.3% in 1995 to 1.1% in 2009, and the seroprevalence of hepatitis B e-antigen (HBeAg) reduced from 5.9% in 1995 to 0.3% in 2009[69]. Furthermore, in Taiwan, the HCC incidence reduced from 0.57 to 0.17 *per* 100000 person-years following mass anti-HBV vaccination[70].

Before the HBV vaccination program, Korea was considered an area of high endemicity. Studies from the 1980s and 1990s revealed that chronic HBV carriage prevalence ranged from 8%-10% before introducing the anti-HBV vaccination in Korea. Since 1990, the percentage of vaccinated infants has surpassed 98.9%, and after 25 years of active vaccination, the HBsAg carrier rate in the general population decreased to 3.7% in 2007. Also, the administration of the anti-HBV vaccine reduced the risk of HCC among adults[71].

However, continuous efforts are needed to ensure timely access to be vaccinated with comprehensive schemes[72]. In spite of the success of vaccination and therapy, chronic hepatitis B (CHB) infection remains a major concern due to many patients ignoring their clinical status. The troubles in diagnosis and screening may be overcome by lifting awareness, favoring partnerships, and allocating resources[73]. In a meta-analysis of 26 studies, the prevalence of HBV infection in non-vaccinated and vaccinated cohorts went from 0.6% to 16.3% and from 0.3% to 8.5%, respectively. The relative prevalence, comparing vaccinated *vs* non-vaccinated, was 0.24 (95%CI: 0.16-0.35) for HBsAg and 0.23 (95%CI: 0.17-0.32) for antibody anti-hepatitis B core antigen. For populations with targeted vaccination, relative prevalence was 0.32 (95%CI: 0.24-0.43) and 0.33 (95%CI: 0.23-0.45), respectively. The residual burden of infection in cohorts offered vaccination suggests that longer-term evaluations of vaccination coverage, timeliness, and other program quality aspects are needed. As HBV-vaccinated infant cohorts reach adulthood, ongoing analysis of prevalence in adolescents and young adults will ensure that elimination efforts are on track[72].

Notwithstanding guidelines suggest screening in high-risk groups like immigrants, these recommendations have not been adopted everywhere[73]. Also, there is a need to improve the uptake of vaccination for household contacts of HBV carriers[74].

The second important strategy to avoid the transmission and control the disease's burden in people living with CHB infection is to guarantee access to medical care and treatment[75,76]. However, most people with CHB live in resource-constrained countries where effective drugs are not always widely available[73]. First-choice drugs in patients with CHB, who meet the criteria for initiating treatment, include nucleoside analogs (ETV) and nucleotide analogs (TDF and TAF)[77-79]. After 10 years of follow-up, TDF and ETV showed effective suppression of the HBV viral load, between 94% and 99%, both in HBeAg-positive and HBeAg-negative patients. HBeAg seroconversion in HBeAg-positive patients with TDF or ETV has been reported in 49%-53% of cases. Alanine aminotransferase normalization has been achieved between 77% and 83% of patients with CHB treated with any of these regimens. However, the annual frequency of HBsAg seroconversion is rare (< 1% annually)[80]. TAF is as effective as TDF but with a better bone and renal safety profile[81-84]. However, some disparities in the opportunity to access hepatitis B therapy have been reported. Miquel *et al*[85] found that a minor proportion of non-immigrants with the indication of effectively receiving hepatitis B therapy got it, compared with non-immigrants (57.8 *vs* 83.2%, P < 0.001)[85]. Similarly, other studies also have reported that immigrants are lost more frequently during the 1st year of follow-up[86]. Immigrants constitute a vulnerable group that would benefit from a more active approach to recognize timely HBV infection and access treatment programs[87].

THE EFFORTS TO CONSTRUCT MICRO AND MACRO-ELIMINATION PROGRAMS THROUGHOUT THE WORLD

The high chronic hepatitis prevalence groups should be recognized and prioritized for detection and linkage to healthcare to reduce the risk of transmitting these infectious diseases. The most vulnerable groups are prison inmates, homosexual men, intravenous drug users (IDU), and sex workers[88]. According to the study by Alonso *et al* [88], in Latin America and the Caribbean, the estimated pooled regional anti-HCV prevalence for IDU was 49% (95%CI: 22.6%-76.3 %); for homosexual men was 3% (95%CI: 1.7%-4.5%); for sex workers was 2% (95%CI: 1.0%-3.4%)[88].

In Canada, penitentiary test-and-treat programs could achieve the most significant decreases in incidence (48%; 95% crude incidence: 38%-57%) over 2018-2030 and prevent the newest first chronic infections (22%; 95% crude incidence: 16%-28%) within those who never exposed to HCV[89]. The project HIPPOCRATES is an example of a micro-elimination program conducted in prison inmates, a vulnerable population to receive treatment less frequently due to many obstacles in healthcare access. The onsite evaluation and treatment of HCV-infected prison inmates achieved an unprecedented effective success rate (SVR was 99%). This type of integral program should be replicated to favor hepatitis C elimination[90].

More attention should be paid to the risk group of homosexual men since HCV incidence in this high-risk group seems to be increasing. In France, a recently important change in HCV epidemiology was reported within HIV-infected patients since the higher rate of HCV transmission occurs in 2018 among homosexual men. From 2012 to 2018, the HCV prevalence among new HIV cases increased from 1.9% to 3.5% in homosexual men. Recently acquired HCV incidence increased from 0.36/100 person-years to 1.25/100 person-years in homosexual men. If well, the proportion of all viremic patients reduced from 67.0% to 8.9%, homosexual men became the first group of viremic patients in 2018 (37.9%), and recently acquired hepatitis represented 59.2% of viremic homosexual men in 2018. Global DAA treatment prescription went from 11.4% to 61.5%. More treatments were initiated in homosexual men in 2018 (41.2%). In homosexual men, treatment at the acute phase represented 30.0% of treatments in 2018[91]. In Spain, a very close to HCV elimination country, homosexual men also carry the highest HCV acquisition risk. The identified main risk factors contributing to new cases of HCV infection in Spain are history of sexually acquired infections [incidence rate ratio (IRR) = 18.2, 95%CI: 1.9-172.1; P = 0.01)], male gender (IRR = 8.3, 95%CI: 1.4-54.2; P = 0.03)] and sharing chem-sex drugs (IRR: 4.9, 95%CI: 1.2-20.8; P = 0.03)[92]. In the Netherlands, homosexual men also have the highest incidence and the highest HCV reinfection rate despite universal and unrestricted access to DAAs, stressing the need for additional preventive measures[93,94].

However, other risk factors should not be minimized either; for example, the unapparent parenteral transmission, through shared nail clippers, rakes, and manicure scissors can also be the primary source of viral infection[95]. Therefore, it is now recommended to perform universal one-time in-life routine HCV screening for all

adults[15].

Likewise, the telemedicine programs and telementoring approaches are outstanding options that may help reduce urban-rural disparities, facilitate access to healthcare systems to receive timely therapy to all kinds of patients who need it, and save costs [96-104]. In Mexico, with the aid of a telemedicine approach, significant savings were achieved by minimizing costs since nearly half of the patients were outsiders. Coverage reached 86%, and treatment with DAAs achieved 99% of SVR[100] (see Table 1).

HOW HAS THE COVID-19 PANDEMIC AFFECTED THE WHO'S GOALS TO ELIMINATE CHRONIC VIRAL HEPATITIS?

Quarantine and social distancing for COVID-19 can drastically affect some parts of the HBV[105] and HCV elimination programs, such as diagnosis, treatment, and harm reduction programs. Therefore, the rate of diagnosis has decreased as voluntary activities such as the NoHep program have been reduced. Furthermore, the incidence of viral hepatitis may increase due to the closure of harm reduction centers[106]. According to the World Hepatitis Alliance global survey to evaluate the collateral damage of the pandemic on viral hepatitis elimination programs, civil society organizations are a vital contributor to the success of the elimination programs; of them, 123 of 131 (94%) reported that the effect of the COVID-19 pandemic altered their activities. A participant from the United States reported that collateral effects from the COVID-19 pandemic included the limitation or even the stop of presential interventions, also affecting community education and detection programs. As a negative outcome, fewer people living with viral hepatitis are expected to be diagnosed during 2020[107]. The World Hepatitis Alliance survey data show that treatment access has been significantly deteriorated by COVID-19 in low-income and middle-income countries (LMICs), with 15 (52%) of 29 respondents from those countries described that the patients could not timely access treatments. However, in high-income countries, like the United Kingdom, the impact of COVID-19 on HCV treatment will be lesser, partly due to telemedicine and home delivery of medicines, conditions that are not very feasible in LMICs[108]. Sperring *et al*[109] explored the impact of the COVID-19 pandemic on screening HCV testing, finding a comprehensive hospital-wide HCV testing reduced by 49.6%, and new HCV+ patient identification reduced by 42.1%. In ambulatory clinics, testing reduced by 71.9%, and new HCV+ identification reduced by 63.3%[109].

According to the mathematical model projection by Blach *et al*[110], a 1-year delay in viral hepatitis elimination programs will result in 44800 [95% uncertainty interval (UI): 43800-49300] excess HCC cases and 72300 (95%UI: 70600-79400) excess liver-related deaths, relative to the no-delay scenario globally, from 2020 to 2030. Most missed treatments would be in LMICs, whereas most excess HCC and liver-related deaths would be among high-income countries. Authorities should privilege hepatitis programs as soon as safe to attenuate the negative impact on elimination programs and reduce excess mortality from delayed treatment[110].

With the approval of a severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) vaccine, most of the possibility to reactivate elimination viral hepatitis programs throughout the world will rely on SARS-CoV-2 effective vaccination strategies that gradually allow restarting the function of viral hepatitis detection campaigns, safe-needle programs, and outpatient clinics to dispenser antiviral medication. According to mathematical modeling analyses, a vaccine with efficacy (VE) ≥ 70% can prevent the infection. A vaccine with VE < 70% may still control the infection transmission if it reduces infectiousness or infection duration among those vaccinated who acquire the infection if it is supplemented with a < 20% reduction in contact rate complemented with herd immunity. The probability of a significant outbreak is zero at VE ≥ 70% regardless of the number of virus introductions. However, an increase in the social contact rate among those vaccinated (behavior compensation) can undermine vaccine impact[111]. Existing reports of currently approved SARS-CoV-2 vaccines indicate their effectiveness at around 95%, making it very plausible to achieve collective herd-acquired immunity based on the mass implementation of vaccination programs against COVID-19 soon[112].

Table 1 World Health Organization's goals to achieve viral hepatitis elimination and strategies to make it

Goal to 2030	Existing resources	Barriers	Strategies that should be improved
Hepatitis C			
90% reduction of new viral hepatitis infections	Harm reduction programs: Safe-sex, safe-needles, and safe-syringes	If well, programs exist in the real-life world are not always sufficiently implemented	Target high-risk population such as MSM, prison inmates, sexual workers, patients with HIV, IDU, immigrants, children born from an HCV+ mother
To reach 90% of patients with viral hepatitis infections being diagnosed	Tests with high sensitivity	If well, detection campaigns exist, it is not enough to reach all people in a real-life setting	Once in life, universal screening for all adults. Also target high-risk population such as immigrants, MSM, prison inmates, sexual workers, patients with HIV, IDU, children born from an HCV+ mother
65% reduction in liver-related deaths	DAAs. Telemedicine and telementoring programs	Still, there is limited access to therapy. More restrained access in LMICs. Vulnerable groups with high prevalence and incidence of viral hepatitis have restricted access to therapy	Flexible policies that guarantee timely access to treatment to all who need it, including vulnerable groups such as immigrants, prison inmates, sexual workers, patients with HIV, IDU, children born from an HCV+ mother when appropriate. Consider including those without healthcare insurance to cover their medication. Encourage telemedicine programs to access communities of difficult access
Hepatitis B			
Prevention of new HBV infections through vaccination and blood safety	Effective and safe vaccine	In the real-life world they are not always available or schemes are applied incompletely	Programs that effectively ensure universal and complete schemes of vaccination at birth for infants and later for those who did not receive the vaccination in childhood. Coverage should be extended and also prioritized for vulnerable groups
Identification, linkage to care, and treatment of persons with chronic HBV	Serologic HBV panels. Nucleos(t)ide analogs with a highly effective and high barrier to resistance Telemedicine and telementoring programs	Serologic HBV panels for diagnosis sometimes are restricted to specialists. Still, there is limited access to therapy, more restrained in LMICs. Vulnerable groups with high prevalence and incidence of viral hepatitis have restricted access to therapy	Basic diagnostic tests (HBsAg and anti-HBc) should be available at primary healthcare. More flexible policies that guarantee timely access to treatment to all who need it, including vulnerable groups such as immigrants, prison inmates, sexual workers, IDU, children born from an HCV+ mother when appropriate. Consider including those without healthcare insurance to cover their medication. Encourage telemedicine programs to access communities of difficult access

anti-HBc: Antibody against hepatitis B core antigen; DAAs: Direct antiviral agents; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HCV+: Positive to hepatitis C virus; HIV: Human immunodeficiency virus; IDU: Injecting drug users; LMICs: Low and middle-income countries; MSM: Men who have sex with men.

CONCLUSION

Chronic viral hepatitis and its complications, cirrhosis, and HCC affect many people worldwide. Without a plan of action, the projection to 2040 will exceed the related mortality as a whole from other significant infectious healthcare problems. Asia-Pacific, Middle East, and North Africa regions have the highest prevalence, representing a substantial burden of the disease. Hopefully, notable advances have been made to achieve WHO goals to 2030 regarding eliminating hepatitis infection better adaptable to actual reality. In that case, actions need to continue being implemented, which must include more harm limitation programs and timely therapy access for those at risk of transmission are certainly needed to reach an incidence decrease. Since universal vaccination is the essential strategy to prevent HBV transmission, continuous efforts are needed to ensure timely access to be vaccinated with comprehensive schemes. Strategies to find positive contacts ensuing a timely screening and diagnosis must be continuously promoted. To avoid viral hepatitis transmission and control the burden of the disease, guarantee access to medical care and effective therapies must include all people who need it, with more emphasis on including vulnerable groups with currently limited access like immigrants, prison inmates, and sex workers. More attention should be paid to the risk group of men who have sex with men since HCV incidence in this high-risk group seems to be increasing. Telemedicine and telementoring approaches facilitate access to healthcare systems and save costs; therefore, this kind of program should be implemented. Finally, the COVID-19 pandemic is currently a significant challenge to achieve viral hepatitis elimination; with the recent approval of a SARS-CoV-2 vaccine, most of the possibility to reactivate elimination viral hepatitis programs throughout the world will rely on SARS-CoV-2 effective vaccination strategies that gradually allows restarting the operativity of liver clinics and services.

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REVIEW

Viral hepatitis update: Progress and perspectives

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Abstract

Viral hepatitis, secondary to infection with hepatitis A, B, C, D, and E viruses, are a major public health problem and an important cause of morbidity and mortality. Despite the huge medical advances achieved in recent years, there are still points of conflict concerning the pathogenesis, immune response, development of new and more effective vaccines, therapies, and treatment. This review focuses on the most important research topics that deal with issues that are currently being solved, those that remain to be solved, and future research directions. For hepatitis A virus we will address epidemiology, molecular surveillance, new susceptible populations as well as environmental and food detections. In the case of hepatitis B virus, we will discuss host factors related to disease, diagnosis, therapy, and vaccine improvement. On hepatitis C virus, we will focus on pathogenesis, immune response, direct action antivirals treatment in the context of solid organ transplantation, issues related to hepatocellular carcinoma development, direct action antivirals resistance due to selection of resistance-associated variants, and vaccination. Regarding hepatitis D virus, we describe diagnostic methodology, pathogenesis, and therapy. Finally, for hepatitis E virus, we will address epidemiology (including new emerging species), diagnosis, clinical aspects, treatment, the development of a vaccine, and environmental surveillance.

Key Words: Viral hepatitis; Hepatitis A virus; Hepatitis B virus; Hepatitis C virus; Hepatitis D virus; Hepatitis E virus

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Core Tip: Viral hepatitis is a global public health concern that affects millions of people and causes thousands of deaths due to acute and chronic infections, cirrhosis, and liver cancer. Although clinical and epidemiological characteristics of hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis D virus, and hepatitis E virus infections are widely known, there are still other critical points that need to be discussed. This review focuses on the most important research topics, dealing unsolved issues and future research directions that can maximize practical impact in the field of viral hepatitis.

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INTRODUCTION

The term viral hepatitis refers to liver inflammation related to a viral infection. As of today, five viruses (hepatitis A, B, C, D, and E) that selectively infect the liver, usually by different routes, have been recognized. In some of these viral infections, acute hepatitis can resolved without intervention, whereas, sometimes, the process turns into a chronic infection[1]. Huge medical advances made in recent decades led to the implementation of preventive measures, the development of vaccines and passive immunization strategies, and, more recently, the development of promising and effective treatments, at least for some forms of viral hepatitis. The results obtained by basic research on viruses and on viruses-cell interaction made it possible to struggle with what a century ago seemed an insurmountable scourge on humanity. Achievements in hepatitis prevention and treatment are perhaps the paradigm of successful translational research[1]. Nonetheless, viral hepatitis is still a global public health concern that affects millions of people and causes thousands of deaths due to acute and chronic infection, cirrhosis, and liver cancer[1,2]. This review focuses on the currently most important research topics and future research directions that can maximize practical impact in the field of viral hepatitis. Table 1 summarizes the principal characteristics of these hepatotropic viruses and Tables 2-6 highlight the main topics of viral hepatitis addressed in the present review.

HEPATITIS A VIRUS

According to the World Health Organization (WHO), 1.4 million new cases of hepatitis A are reported worldwide each year, with a consequent nearly 7000 deaths[3]. Hepatitis A virus (HAV), a member of the *Picornaviridae* family and the only species from the *Hepatovirus* genus that infects humans, is a non-enveloped single-stranded RNA virus[4]. HAV is classified into six genotypes, three infecting humans and three affecting simians, but there is only one known serotype[5].

Despite HAV being discovered more than 4 decades ago, it has been well characterized, and its detection and diagnosis have been widely implemented; changes in the socio-economic conditions and the control mechanisms of the virus have triggered new circulation and transmission scenarios that have generated new targets for its assessment. Some of them are the epidemiology and molecular surveillance of the virus, the different vaccination schemes and immune responses, the new susceptible populations (after the implementation of massive vaccination), and the study of the virus in environmental and food matrices.

Epidemiology and transmission: Old and new challenges

Although HAV epidemiology is complex, it is changing in those countries that are improving their public health and sanitation policies, considering that the most usual routes of HAV transmission are contaminated water ingestion and the contact with infected individuals[6]. Three circulation patterns have historically been described for

Table 1 Features of different types of hepatitis virus

	HAV	HBV	HCV	HDV	HEV
Family	*Picornaviridae*	*Hepadnaviridae*	*Flaviviridae*	Undefined[1]	*Hepeviridae*
Genus	*Hepatovirus*	*Orthohepadnavirus*	*Hepacivirus*	*Deltavirus*	*Orthohepevirus*
Genome	Positive single-stranded linear RNA	Double stranded gapped DNA	Positive single-stranded linear RNA	Negative single-stranded circular RNA	Positive single-stranded linear RNA
Genome length (kb)	7.5	3.2	9.6	1.7	7.2
Genotype	6 genotypes: I, II and III infect humans, and IV, V and VI infect non-human primates	10 genotypes (A to J)	8 (1 to 8)	8 (1 to 8)	8 (1 to 8)
Transmission	Fecal-oral	Parenteral, sexual, and perinatal	Exposure to infected blood	Exposure to infected blood and body fluids	Fecal-oral; zoonotic; blood transfusion
Treatment	None. In case of severe hepatitis, treatment of symptoms	Pegylated interferon-alpha and nucleoside/nucleotide analogues	DAA	Pegylated interferon-alpha	Ribavirin (in chronic HEV infection)
Prophylaxis	Yes (inactivated vaccine)	Yes (recombinant vaccine)	No	Yes (HBV vaccine)	No[2]
Clinical outcome of infection	Self-limited	Self-limited and chronic	Self-limited and chronic	Self-limited and chronic	Self-limited
Chronic infection rate	No	Depends on the age of acquisition of the infection. Birth or in infancy 90%, 1 yr and 5 yr of age 30%-50%, adulthood 5%. Hemodialysis patients 40%. Immune deficient patients 20%	80%	More frequent in HBV/HDV superinfection than coinfection	Acute infection in most of the cases. Chronic infection in immunosuppressed populations

[1]It is not defined yet in any of the established viral families.
[2]There is only one vaccine, approved and used only in China. HAV: Hepatitis A virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HDV: Hepatitis D virus; HEV: Hepatitis E virus; DAA: Direct antiviral agents.

Table 2 Hepatitis A virus highlights

	Hepatitis A virus
1	The risk of HAV infection is associated with the lack of safe water and poor and sanitation
2	Due to the vaccine introduction in childhood, young adults are becoming more susceptible to HAV infections
3	In countries where waterborne transmission is rare, outbreaks occur among men who have sex with men, injecting drug users and contaminated food
4	Since molecular detection is not routinely performed for diagnosis, surveillance programs, including viral amplification and sequencing, are needed to know the strains that circulate in a certain place
5	One of the greatest challenges for HAV is to increase vaccination coverage globally, still implementing the single-dose schedule, to decrease the new infections, and, in the long term, to achieve its eradication

HAV: Hepatitis A virus.

HAV: (1) In high endemicity areas from low- and middle-income countries, where the incidence varies from low to high over time and between different regions, there is a peak age of infection in early childhood that is frequently asymptomatic, the transmission pattern is person-to-person, and outbreaks are uncommon due to high rates of immunity from previous childhood infection; (2) In moderate endemicity areas, from middle-income countries (regions where sanitary conditions are variable), the incidence is high, the peak age of infection is in late childhood/adolescence or in young adults that is frequently symptomatic, the transmission pattern is also from person-to-person, related to food and water, and therefore outbreaks are common due to low rates of immunity from previous childhood infection; and (3) In low endemicity

Table 3 Hepatitis B virus highlights

	Hepatitis B virus
1	Several host factors, such as male gender, alcohol intake, and obesity have been associated to worse disease progression. Current challenge implies finding genetic markers to predict the course of HBV infection. In this line, different SNPs associated with the outcome of HBV infection have been recently identified
2	In the last years, new diagnostic assays have been developed in the framework of the diagnosis of HBV infection. The implementation of quantitative HBsAg, HBcAg, and HBV-RNA in routine clinical practice could probably improve the management of patients with CHB
3	Current antiviral treatments have some shortcomings, such as poor SVR or prolonged schedules. Direct antiviral agents against different HBV targets, including HBV cccDNA, are under evaluation. Moreover, immunemodulatory therapies to overcome host immune impairment observed in chronic infections are being investigated
4	Although a safe and cost-effective vaccine is available since the 1980s, an inadequate response is achieved in particular settings. New and more potent adjuvants, as well as formulations that include alternative viral antigens could improve the response rate vaccination
5	The development of new antiviral therapies that enables achieving functional cure as well as accurate diagnostic methods and more effective vaccines will contribute with the purpose of the WHO to eliminate by 2030 hepatitis as a global health problem

HBV: Hepatitis B virus; SNP: Single nucleotide polymorphisms; HBsAg: Hepatitis B surface antigen; HBcAg: Hepatitis B core Antigen; CHB: Chronic hepatitis B; SVR: Sustained virological response; WHO: World Health Organization.

Table 4 Hepatitis C virus highlights

	Hepatitis C virus
1	WHO global hepatitis elimination strategy aims to reduce 90% of new HCV incidence, 65% of mortality and treat at least 80% of patients
2	DAA treatment leads to regression of clinical symptoms and liver disease complications even in those patients with other comorbidities, co-infections, or advanced liver disease
3	The immune response plays a central role in viral elimination. The understanding of the relationship between achieving protection and activation of immune responses is mandatory for the development of an effective prophylactic vaccine
4	Immune response restoration after DAA treatment is also under debate, certain immune features are reinvigorated, but many immune exhaustion signs may persist
5	SVR after DAA rates higher than 97% are usually attained, but still, a minor group of patients (4%-5%) fails to eradicate HCV due to resistance-associated variants, some of them arising after treatment but others naturally occurring in treatment naïve individuals
6	DAA efficacy impacts on transplantation from HCV-infected donors into infected or uninfected recipients; however, early outcome data are encouraging, experience is limited, and many issues remain under debate
7	HCC risk after DAA treatment has been extensively discussed; however, recent seminal reports support the notion of a reduced rate for occurrence or recurrence of HCC after DAA SVR
9	There are numerous HCV vaccine approaches including a few candidates who accomplished phase I trials, but a prophylactic HCV vaccine that can contribute to the eradication goal remains a pending issue

DAA: Direct antiviral agents; SVR: Sustained virological response; WHO: World Health Organization; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

areas from high income-countries, the incidence is low, the peak age of infection is in young adulthood, the transmission pattern is from person-to-person and also *via* food and water; and outbreaks are common due to low rates of immunity from previous childhood infection[3].

Nowadays, 34 countries have included vaccination against HAV in routine immunization programs among children[3]. Many countries use an inactivated HAV vaccine with a two-dose regimen, while other countries have successfully implemented it in their immunization programs in a single-dose[3,7]. However, long term results of the single-dose schedule have been only partially studied. The most recent investigation showed sustained immunologic protection for up to 9 years, with high levels of antibody titers, when children were vaccinated at 12 mo[8]. More studies that assess long-term seroprotection against HAV after single-dose vaccination scheme are necessary to monitor the effectiveness of this innovative strategy. In some territories vaccination is also recommended for people at risk of HAV infection, like those who travel to regions where HAV is endemic, drug users, men who have sex with men, and individuals with chronic liver disease[3].

The recent improvement of socio-economic, hygienic, and sanitation measures may translate into an increase in the number of adults who have never been infected in

Table 5 Hepatitis D virus highlights

	Hepatitis D virus
1	The natural course and outcome of acute hepatitis D differ according to HBV and HDV co-infection or superinfection
2	HDV and HBV genotypes in addition to host factors influence the course of chronic hepatitis
3	The implications on liver disease of HVD, HBV, and innate immunity interplay remain to be understood
4	Chronic setting leads to more severe hepatitis associated with higher rates of HCC and a faster progression to cirrhosis compared with HBV monoinfection. HDV pathologic changes are limited to the liver with histopathologic features that are not specific for it
5	HDV remains difficult to treat with the current available therapies, and although, several promising new therapies have been described treatment is still the greatest challenge in HDV infection

HDV: Hepatitis D virus; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma.

Table 6 Hepatitis E virus highlights

	Hepatitis E virus
1	HEV is transmitted by the fecal-oral route (involving contaminated waters) and also as a zoonosis
2	In the last years, many studies have focused on HEV detection in environmental and food matrices, and blood products as alternative sources of infection
3	A new etiological agent of human hepatitis E, Orthohepevirus C, previously known to infect rats, has been recently described
4	Although most cases of HEV infection produce acute hepatitis, chronic infections seem to be an increasing problem, particularly in Europe
5	Complications and extrahepatic manifestations are also increasingly recognized
6	Only one vaccine for HEV has been licensed in China, with little known data, which limits its use

HEV: Hepatitis E virus.

childhood and therefore lack immunity. Furthermore, despite pediatric immunization programs, many young adults may have been above the cut-off ages to be included when such social programs were introduced. Therefore, young adults are now becoming more susceptible to HAV infections, so in areas of low and middle-endemicity, the prevalence of symptomatic cases in this age group has increased[6]. In this sense, between the middle of 2016 and the beginning of 2018, several hepatitis A outbreaks were reported in Europe, the United States and South America, which disproportionately affected HAV unvaccinated young adult men, mainly men who have sex with men. This group presents an increased risk of infection same as persons, regardless sex orientation, who have oral-anal sexual contact[4]. Interestingly, through phylogenetic analysis accompanied by detailed questionnaires to capture the sexual history of the patients, it was possible to establish epidemiological links between cases, demonstrating that the viruses responsible for these outbreaks belonged to HAV genotype IA and grouped with one of these strains: VRD_521_2016, RIVM-HAV16-090, and V16-25801[6]. This highlights the importance of carrying out a more detailed epidemiological record of cases, including sexual history, which will help to establish the source and chain of infection.

Regarding travelers to endemic regions, although the WHO has recommended their vaccination, it does not always happen, increasing hepatitis A cases among this group. Furthermore, the movements of immigrants in some areas of the world (*e.g.*, in South America) led the virus to be transported from endemic areas (often without vaccination coverage) to non-endemic areas, introducing new viral strains[3,6]. Screening for immunoglobulin (Ig) G anti-HAV should be offered to this group; therefore, patients who test negative should be offered vaccination[4].

Detection and surveillance

Diagnosis of hepatitis A is performed by the detection of HAV IgM with serological assays. Specific antibodies are present in sera for at least 7 mo after infection, although in some individuals they remain for up to a year[4]. During acute infection, IgG anti-HAV appears, and it remains present in serum for life[3,4]. Serological surveillance is assumed as the main monitoring strategy for the infection. Since molecular detection is

not routinely performed for diagnosis, surveillance programs, including viral amplification and sequencing, are needed to understand the strains that circulate in a certain place or that are introduced by travelers; however, it is seldom carried out. Molecular surveillance includes the detection and study of HAV in environmental and food matrices, an area of study that has been carried out in recent years. For the purpose of molecular surveillance, the HAV Network (HAVNET) was created in 1999 [9]. This is an international HAV network of scientists who work in reference laboratories of hepatitis A and share molecular and epidemiological data on this virus, information that is useful for the scientific community. The HAVNET aims to increase the knowledge of HAV infections and map the worldwide distribution of HAV strains. As there is a strong geographical signal in the sequences, this can be used for source tracking.

The study of HAV in environmental and food matrices is a valuable tool for monitoring circulating HAV strains, to know the sources of infection and to take sanitation and prevention measures. After a large outbreak of foodborne hepatitis A in Europe in 2013-2014, the crucial role of sequence data analysis to investigate outbreaks and define transmission pathways was recognized, as well as the need of the agreement on a common genomic region for sequencing and a common protocol to perform HAV detection in food[10]. In this sense, with the aim of harmonizing the existing protocols for HAV detection in food, the European Committee for Standardization and the International Standards Organization developed and published a standard methodology for quantitative and qualitative determination of HAV (together with norovirus) in seven food matrices, using real-time (RT) polymerase chain reaction (PCR), which has allowed to obtain comparable results between laboratories[5]. Furthermore, the sequencing of a common consensus region was agreed to target the HAV VP1/2A junction and thus promote the protocol described in the HAVNET[10]. In this context, collaborations between the public health sector, the food sector, HAVNET, and other organizations, together with government dependencies, are highly recommended.

Although there is no legislation about the presence of HAV in environmental matrices at a global level, some countries have adopted measures for the surveillance of cases of food outbreaks due to HAV, which has led to strict controls of imported food, incorporating the mandatory control of this virus in some cases[11]. Foodborne HAV clinical cases and outbreaks are difficult to identify, track, and assess their magnitude due for many reasons: (1) The difficulty for patients to remember food consumption history before the onset of the disease; (2) The asymptomatic nature of many cases, which are not reported (in the case of outbreaks); (3) The long incubation period of HAV; (4) Viral contamination levels of a food item may be low and focal and, therefore, hard to detect; and (5) The scarce knowledge of health care teams about foodborne viral diseases[5].

The above issues highlight the new epidemiological scenarios of this virus, showing the targets to whom control and prevention actions should be directed. The main goal for the next years should be to increase vaccination coverage globally, implementing the single-dose schedule, so to decrease the new infections, and, in the long term, to achieve eradication.

HEPATITIS B VIRUS

The hepatitis B virus (HBV) was discovered by serendipity in the 1960s and subsequently several milestones were achieved such as the development of diagnostic tests in the early 1970s or the implementation, in the 1980s, of a safe and cost-effective vaccine with subsequent different therapies for the treatment of chronic hepatitis B (CHB) infection[12].

Despite these advances, the landscape is still far from satisfactory. Currently, an estimated 257 million people are living with CHB, and around 887000 deaths occur annually as a consequence of infection progression, mainly due to cirrhosis and hepatocellular carcinoma (HCC)[13]. Furthermore, it is expected in the coming decades that the problem of HBV infection might increase, particularly in developing countries, as a consequence of the limited access to diagnosis and treatment, in addition to the subclinical characteristics of the infection[14]. In fact, the WHO has proposed strategies to eliminate viral hepatitis as a Public Health problem by 2030. To achieve this goal, it will be necessary to implement prevention, diagnosis, and treatment measures, as well as to raise awareness among the population and primary care physicians from the infections caused by HBV[15].

Among the current challenges to overcome are the identification of host markers that would allow to predict accurately the evolution of infection and the implementation of a personalized medical approach, the development of anti-HBV therapies that enables achieving functional cure in chronically infected patients, as well as the restoration of the host's immune response, the implementation of new diagnostic methods, and the development of more effective vaccines that would lead to improving prevention policies in order to reduce the global burden of HBV disease.

Host factors

HBV infection has a wide range of clinical presentations, from subclinical to symptomatic in the acute stage, and from inactive carrier state to active chronic hepatitis with different degrees of severity[16]. Epidemiological data early established that the course of the infection is closely related to the age at which the infection is acquired, being the evolution to chronicity much more frequent in individuals infected at birth or in childhood[17]. Additionally, male gender, heavy alcohol consumption (more than 60 g/d), obesity, and comorbidities, such as co-infections with human immunodeficiency virus (HIV), hepatitis C virus (HCV), or hepatitis D virus (HDV), have also been reported to contribute to progression to end-stage liver disease[18-22]. Recently, genome-wide association studies have shown that the host genetic background may also affect the natural history of infection[23,24]. Several studies have identified single nucleotide polymorphisms (SNPs) in human leukocyte antigens (HLA) that have been associated with the outcome of HBV infection, either with clearance or progression of chronic infection, although some findings were not subsequently supported in other manuscripts. Among the more in depth characterized, it has been found that HLA-DP (rs3077 and rs9277535) and HLA-DQ (rs7453920 and rs2856718) SNPs were associated to HBV persistence[25-27] Notably, different studies have also identified several HLA polymorphisms associated with the response to the HBV vaccine[28,29]. Additionally, it was also reported that cytokine, chemokine, toll like receptor, sodium taurocholate cotransporting polypeptide, and vitamin D-related genes may influence the clinical outcomes of HBV infection[24,30,31]. Beyond the controversies observed among studies addressing the genetic polymorphisms involved in the outcome of HBV infection, mainly probably due to ethnic differences (haplotype structures and allele frequencies), these findings will undoubtedly help to individualize the risk of infection progression and to improve the effectiveness of HBV vaccination campaigns, contributing to the implementation of a personalized approach and a greater chance of accomplishing the achievement of eliminating HBV infection as a public health problem by 2030.

Diagnosis

In order to achieve global control of HBV infection, one of the main obstacles to overcome is the limited access to diagnostic resources. Since the 1980s, classical serological markers have been available, including detection of antigens s and e (HBsAg, HBeAg) and antibodies against antigen e and core (anti-HBe and anti-HBc), along with the later use of molecular markers to determine the viral load, for the diagnosis and management of HBV infection. The qualitative detection of HBsAg has been the hallmark of HBV infection. Its presence for more than 6 mo is pathognomonic of chronic infection, and HBsAg seroclearance is now considered the goal for functional cure, except for occult hepatitis B, in which HBsAg is not detected despite the persistence of the infection. In recent years, efforts have focused on the search for accurate tools for the monitoring of antiviral treatment in CHB. Complete cure of CHB infection implies elimination of the HBV from infected hepatocytes, which is hardly achievable because of the persistence of the covalently closed circular DNA (cccDNA) and integrated HBV-DNA. Since cccDNA detection is difficult to perform in routine diagnosis, surrogate markers have been developed, being the quantitative HBsAg (qHBsAg), the hepatitis B core-related antigen (HBcrAg), and serum HBV-RNA the most promising ones. In the last years, different assays to qHBsAg levels have been developed. In most studies carried out on HBeAg-positive patients, a positive correlation among HBsAg titers, serum HBV DNA, and liver cccDNA has been observed[32]. In contrast, this relationship was not verified in HBeAg-negative CHB cases[33]. The lack of correlation could be a consequence of S gene mutations associated with HBeAg seroconversion, affecting expression or secretion of HBsAg[34-36]. Nonetheless, several studies have shown that qHBsAg is a useful diagnostic tool, together with HBV-DNA levels, to discriminate inactive carriers from HBeAg-negative chronic hepatitis[37,38]. Furthermore, it has been described to be useful in predicting sustained HBsAg clearance and liver disease progression in inactive carriers[33]. Likewise, baseline and on-treatment qHBsAg levels have been shown to be a reliable

prognostic marker of sustained virological response (SVR) in treatment with pegylated interferon alpha (PEG-IFN-α). Consequently, current guidelines recommend its use for the management of HBV therapy[39-42]. The HBcrAg, another recently developed marker, detects the HBcAg, HBeAg and the 22 kDa precore protein. Different studies indicate that HBcrAg depicts a more accurate correlation with intrahepatic cccDNA transcriptional activity than qHBsAg, regardless of HBeAg status[43,44]. Furthermore, HBcrAg has been suggested as a prognostic factor for virological remission and HBsAg clearance in patients undergoing antiviral treatment[45], as well as a predictive marker for the development of HCC[46]. Nevertheless, its clinical use remains controversial. HBV-RNA detection has also raised interest as a possible surrogate marker of HBV transcriptional activity since serum HBV RNA levels significantly correlated with intrahepatic cccDNA concentrations among untreated patients[47,48]. Likewise, it has been suggested that HBV RNA has a predictive value as a diagnostic tool of HBeAg loss in patients under therapy, being proposed as a reliable marker for treatment discontinuation[49,50]. However, routine implementation still requires standardization of the methodology. Finally, several studies have identified other promising markers to monitor the management of CHB patients such as quantitative anti-HBcAg or cccDNA determination[51-53]. Further validation for their use in clinical practice is still required.

Therapy

Over the last 2 decades, notable progress has been achieved in the treatment of CHB infection. Currently available antiviral agents include PEG-IFN-α and nucleoside/nucleotide analogues (NAs) among which entecavir, tenofovir disoproxil fumarate, and tenofovir alafenamide are the first-line oral anti-HBV drugs due to the high genetic barrier to HBV resistance. Although suppression of HBV replication reduces the progression of liver disease and improves the outcomes in most patients, the actual obstacle to cure CHB is the persistence of cccDNA and integrated HBV DNA. Thus, the term 'functional cure' has been accepted as the ultimate goal to reach with HBV therapies[41,42]. However, PEG-IFN-α treatment has an unsatisfactory SVR rate in addition to several adverse effects, being therefore limited to a selected group of patients. On the other hand, although NAs have shown high efficacy in inhibiting viral replication, the HBsAg sustained clearance rate is poor, with a substantial risk of relapse when treatment is discontinued, the need for retreatment, and the risk of select drug resistant strains[54]. Therefore, the main challenge at present is the implementation of new strategies that increase the rate of loss of HBsAg or the sustained suppression of HBV replication compared to existing therapies by developing more efficient antiviral agents and immune-modulatory therapies to restore the functionality of the immune system. Direct antiviral agents targeting different HBV proteins or steps of the viral replication cycle are being evaluated. HBV entry inhibitors are molecules that target the NTCP receptor (NTCP: Sodium taurocholate cotransporting polypeptide is the host cell receptor required for HBV entry), preventing both the novo infection and reinfection cycles, being of great value to control CHB infection[55,56]. Also, HBsAg release inhibitors are promising drugs that combined with current antiviral treatments might help to induce HBsAg clearance [57]. Additionally, core protein assembly modulators and small interfering RNA targeting HBV transcripts are under evaluation in different clinical trials[58,59]. Another appealing strategy implies targeting the HBV cccDNA. Several molecules and clustered regularly interspaced short palindromic repeats technology have shown the ability to inhibit synthesis or eliminate the already formed cccDNA[60]. However, they are still under investigation due to delivery issues and unintended off-target effects [61]. Furthermore, antiviral agents against the protein X are also being addressed, both for their role in the epigenetic regulation of cccDNA and in the modulation of several host cell signaling pathways[62].

As mentioned above, the impairment of the host's immune system is another important factor for HBV persistence. Several approaches are being investigated for the pharmacological activation of the intrahepatic innate immune response, including the induction of IFN genes with antiviral properties[63] or the stimulation of toll-like receptors (TLR)[64], targeting adaptive cell effectors. In line with the latter strategy, some attractive methodologies include the use of checkpoint inhibitors that block the co-inhibitory receptors overexpressed in HBV-specific T cells to reverse immune dysfunction[65], the adoptive transfer of either genetically engineered T lymphocytes expressing chimeric antigen receptors or reinfusion of autologous restored T cells[66,67], as well as therapeutic vaccination that might boost host immune response[68]. However, the wide range of functional deficiencies observed in patients with CHB represents an important pitfall for the success of these therapies. Its use together with

antivirals agents is expected to lead to viral elimination as well as mounting strong immunological surveillance that limits viral reactivation.

Vaccine

Vaccination is the most powerful tool to control the spread of HBV. Since the 1980s, a recombinant HBV vaccine obtained by expressing the small envelope protein (HBsAgS) in yeast has been available. It is currently being implemented for infants in more than 189 countries, and in 109 of these a dose within the first 24 h of life has been introduced in vaccination schedules[69]. Following the global introduction of large-scale vaccination, a substantial decrease in the rate of HBsAg carriers was observed [70]. After three intramuscular doses, a protective response against HBV is achieved in more than 90% of healthy adults and more than 95% of infants, children, and adolescents. However, the response rate declines with age, particularly after the age of 40, as well as in people with obesity, smokers, comorbidities, genetic factors, or particular settings[71]. Failure to mount an adequate immune response is one of the main concerns regarding the HBV vaccine; therefore, to overcome this shortcoming, several attempts to enhance the immunogenicity have been addressed. On the one hand, new and more powerful adjuvants have been developed and evaluated, including liposome-based formulations, cytidine-phosphate-guanosine oligodeoxynucleotide (a TLR9 agonist) or virosomes[72]. These reformulations have shown a considerable improvement in seroconversion rates compared to the conventional vaccine, particularly in individuals with poor or no response[73]. Interestingly, the novel adjuvants may reduce the current schedule from three to two doses, contributing to a higher adherence rate to compliance with the vaccination scheme, which is another drawback[74]. Likewise, intradermal administration has shown to provide better responses than the intramuscular route[75].

On the other hand, recombinant vaccines derived from mammalian cells containing the medium and large envelope proteins, in addition to the already used small envelope protein, have been developed. This approach has the advantage of antigens displaying the same post-translational modifications and protein folding that occurs *in vivo*. This alternative approach showed a faster seroprotection rate as compared to the conventional vaccine, making it of particular interest for people with poor or no response. Furthermore, it could protect against HBV strains carrying vaccine induced or spontaneous HBsAgS mutants[76]. In fact, the emergence of vaccine escape mutants (VEMs) and the role of the HBV genetic variability both have been considered as possible shortcomings of the vaccine.

Shortly after the massive implementation of the HBV vaccine, the selection of variants with mutations in the wild type epitope has been reported. In many countries, where early large-scale vaccination was introduced, along with a decrease in the prevalence rate of infection, over time a significant increase in the frequency of VEMs [77] has been observed. Different studies have shown that VEMs can replicate, with the implicit risk of becoming the predominant strains in the coming decades. In addition to the selection pressure exerted by the implementation of large scale vaccination, due to the overlapping of the open reading frames in the HBV genome, mutations in the *Pol* gene can affect the *S* gene[78]. Consequently, the use of antiviral agents targeting the viral polymerase indirectly promotes the selection of mutants affecting the HBsAgS recognition by vaccine-induced antibodies. Beyond these assumptions, the transmission of VEM is a very unusual event and, although its strict vigilance is recommended, it does not pose a threat to the control of HBV infection. Therefore, the introduction of mutated antigens in the vaccine formulation is not currently being considered[79].

Finally, the genetic variability of HBV may represent a more significant problem than VEMs. Based on the genetic diversity, HBV is classified into 10 genotypes (A to J) and several subgenotypes. The HBV vaccine used today was developed decades ago, when the existence of the different HBV genotypes was unknown, using HBsAgS of genotype A2 as a prototype. Case reports of vaccinated people subsequently infected, mainly with the most divergent genotypes, have been described[80-82]. Although there is a paucity of data regarding cross-genotype preventive effect, greater protection against homologous genotype/sub-type than against heterologous strains of HBV have been reported[83]. However, empirical data from regions where the most divergent genotypes are prevalent suggests that cross protection is sufficient to prevent infection. Therefore, HBV diversity would not represent a major obstacle to the prophylaxis of infection.

HCV

In 2020 the Nobel Prize in Physiology or Medicine was awarded to the Americans Harvey J Alter (United States National Institutes of Health) and Charles M Rice (Rockefeller University) and to the British Michael Houghton (University of Alberta) for the discovery of the HCV. Alter demonstrated the existence of a non-A non-B hepatitis virus-associated with post-transfusion hepatitis in 1975, Houghton cloned and identified the viral genome and renamed it as HCV in 1989, and Rice established, from an edited version of the virus genome, a robust *in vitro* replication system in cell cultures in the 1990s and thus laid the foundation for future genetic and functional analysis[84-86].

Epidemiology and treatment

HCV is an enveloped single-stranded RNA virus of the *Flaviviridae* family. Due to a lack of proofreading activity of HCV RNA-dependent RNA polymerase (NS5B) and their high replication rate, a large number of viral variants are produced during infection[87]. Eight genotypes have been described, among them genotype 1 is prevalent worldwide, while the others were each characterized in different geographic regions, that is genotype 2 in West Africa, genotype 3 mostly in South Asia, genotype 4 in Central and North Africa, genotype 5 in South Africa, genotype 6 in South-East Asia, and genotype 7, which has been isolated from central African immigrants in Canada[88,89]. Recently, the novel genotype 8 was described as endemic in India[90].

HCV is estimated to infect more than 1% of the global population, and around 80% develop a slowly evolving, asymptomatic chronic liver disease characterized by cell damage, inflammation, and fibrosis that can progress, after a few decades, to cirrhosis in 30%-40% of cases or to HCC in 1%-3% of them[91]. Thus, HCV infection is strongly related to liver transplantation. In the absence of a vaccine, HCV treatments went through different stages, starting with prolonged regimens based on interferon as an immune system modulator with cure rates of less than 50% and high adverse effects, and going through successive generations of direct-action antivirals (DAA). However, the real improvement of the DAA regimen began in 2013, when an interferon-free treatment was available; since then, several DAA schemes targeted against the protease, the NS5A protein, or the polymerase became the standard of care. Currently, treatments are oral with almost no side effects and with SVR rates higher than 97% after 8 to 12 wk. Nowadays, successful treatment leads to regression of clinical symptoms and complications of liver disease even in those patients with other comorbidities, co-infections, or advanced liver disease[92-94]. In this new scenario, as mentioned above, in 2016 the World Health Assembly approved a global strategy to achieve viral hepatitis elimination (C and B), which concerning HCV aims to reduce 90% of new HCV infections (incidence), 65% of deaths (mortality), and treat at least 80% of patients who require treatment[95,96]. However, this objective is far from being reached. On the one hand, it is critical that each country implements systematic and organized programs of silent carrier detection to overcome the suboptimal rates of HCV screening. On the other hand, it is necessary to ensure access to treatment for all infected people, which is still difficult due to the high cost of it. Finally, it is essential to carry out primary prevention tasks to avoid the generation of new cases and the reinfection of patients already cured, especially in the groups at greatest risk[95,96].

Pathogenesis and immune response

Chronic hepatitis C pathogenic mechanisms as well as the immune response participation in the generation of liver damage are still topics of interest[91,97]. It has been thoroughly described that HCV alters liver homeostasis, leading to stress and inflammation[98]. The liver microenvironment is extremely complex with numerous immune cell populations that, along with the cytokines that they produce, play a central role in the viral elimination; thus the interplay between virus and host immune response may influence infection outcome[99-101]. Remarkably, in the chronic stage, the role of the immune cells becomes more complex since their altered functionality would contribute to liver damage[102]. Cellular immune surveillance of HCV infection induces interferon production and activates innate immune response, hence controlling the infection. As part of the innate immune response, natural killer, dendritic, and Kupffer cells present viral antigens from infected hepatocytes to the T and B lymphocytes, which in turn contribute to virus control. The immune system triggering is not enough to control HCV infection and in consequence, a persistent infection is established. However, the results behind this data are controversial, and the underlying mechanism by which various cell populations are involved is still

under discussion.

The understanding of the relationship between achieving protection and the activation of both innate and adaptive immune responses is mandatory for the development of an effective prophylactic vaccine that may control infection and transmission. Moreover, the restoration of the immune response after DAA treatment is also under debate, particularly because most reports have focused on immune cells in peripheral blood, and little is known related to the intrahepatic immune environment after rapid clearance of chronic HCV. Remarkably, a double scenario arises after DAA treatment, certain immune features are reinvigorated but many immune exhaustion signs may continue after viral elimination[103]. The majority of pro-inflammatory cytokines and chemokines reached normal values after long-term monitoring albeit IFN-α and tumor necrosis factor-related apoptosis-inducing ligand maintained high levels for months after treatment[104,105]. Regarding HCV-specific T cells, a partial recovery of the functionality, mainly proliferation capacity, was described. Nevertheless, it does not apply for every patient, and the restoration level was not homogeneous for all individuals. The suppression of HCV replication led to a decrease in expression of T lymphocyte exhaustion markers and an increase in HCV-specific IFN-γ responses after treatment[94,106,107]. However, the restoration of exhausted HCV-specific CD8+ T lymphocyte surface phenotype does not result, *per se*, in a complete functional restoration. Regarding CD4+ T cells, HCV antiviral treatment leads to a shift from a T helper 1 cell to a follicular helper T cell (Tfh) environment within HCV-specific cells. Likewise, HCV-specific CD8+ T lymphocytes, Tfh cells are likely to persist in an antigen-independent manner[108]. Furthermore, in chronic hepatitis C, regulatory T cells are usually elevated and display an activated phenotype in the course of infection that persists even after DAA therapy[109]. Natural killer (NK) cells have an important role in HCV infection control; however, phenotype and function of NK cells are altered in chronic HCV patients[110]. In recent years, several groups have investigated the recovery of the altered NK cell compartment upon successful antiviral treatment, but it is still a matter of research whether an active reinvigoration *via* certain signaling pathways or the resolution of inflammation after virus elimination are responsible for a seemingly restored NK cell compartment. Hence, such a persistent challenge of the immune system might trigger irreversible damage that in turn could affect the success of any therapeutic vaccine design or even any immunotherapy approach against HCC[94,103,111].

DAA resistance-associated variants

Current DAA therapy has demonstrated high efficacy, but still in a minor group of patients (4%-5%) it does not succeed in eradicating HCV, largely due to inadequate adherence but also due to relapse or viral fitness[112]. Since HCV is a rapidly evolving RNA virus, the exposure to DAAs triggers strong drug selection favoring mutants that offer partial resistance to them. Thus, the high SVR achieved with DAA still faces the challenge of resistance-associated variants (RAVs), some arising after treatment but others naturally occurring in treatment naïve individuals[113,114]. DAA treatment failure may be attributable to advanced liver disease, suboptimal therapy adherence, and the presence or generation of NS5A mutations[112]. The three HCV non-structural proteins have different RAV prevalence, which may be related to their distinct roles in the HCV life cycle that defines the resistance genetic barriers[115]. RAVs affecting each of the DAA classes have different properties and occur most commonly in the NS5A region, less commonly in the NS3 region, and uncommonly in the NS5B region. In all first-line DAA regimens, NS5A inhibitors are a crucial component because their RAVs have direct clinical impact[114,116]. Treatment-emergent RAVs that remain at high frequency after the end of therapy often have other fitness compensating mutations and may be more difficult to treat. Nowadays, there are several options for patients who have failed to respond to first line DAA therapy, and more than 90% of these patients are able to achieve SVR following retreatment, but the selection of the appropriate therapy depends on several factors and may require genotype and resistance testing. It should be noted that there are some notable genotype-specific differences with respect to retreatment, particularly in the case of prior exposure to NS5A inhibitors in patients with genotype 1 infection. Rescue treatment options with multiple targeted therapies, such as the pangenotypic combinations, sofosbuvir/velpatasvir/voxilaprevir (Vosevi), and glecaprevir/pibrentasvir (Mavyret), were effective in the majority of cases with DAA failure[112]. Interestingly, a recent European multicentric study showed that even Vosevi can fail in genotype 3 and genotype 1a infected individuals with cirrhosis, but this failure is not associated with a specific pattern of RAV. It is important to note that rescue treatment with multiple targeted therapies was effective in the majority of patients[117].

DAA treatment in the context of solid organ transplantation

The field of solid organ transplantation (SOT) has also been benefited with DAA development[118,119]. The efficacy of DAA has created a new opportunity to improve survival in end-organ failure patients through greater access to organ transplantation, since transplanting organs from HCV-infected donors into infected or uninfected recipients is now under consideration. Altogether, this has led to a better transplant outcome due to healthier patients receiving SOT and a significant reduction of waitlist mortality and healthcare costs. In the post-liver transplantation setting, early treatment is now recommended due to the high efficacy of DAAs, in association with a low side effect profile and easily mitigated drug-drug interactions[118,119]. The optimal treatment duration for each organ is not yet clear; in the years to come there will be increasing data and hopefully standardization of treatment. Some reports proposed 8-12 wk of DAA treatment for liver transplantation, but 2-4 wk seems to be enough for other organs[118,119]. However, it should be kept in mind that although it is expected that treatment eliminates the risk of infection, this is not a certainty; since the persistence of HCV RNA in peripheral blood mononuclear cells and/or the liver has been shown to occur post-SVR in liver transplantation recipients, with unclear clinical consequences. Although early outcome data are encouraging, the overall experience is limited, and many ethical issues and scientific questions remain, such as avoidance of selection bias, the optimal timing of DAA therapy, detailed evaluation of drug-drug interactions between DAAs and immunosuppressants, and long-term graft-patient outcomes. Moreover, there is no data on possible long-term hepatic and extrahepatic adverse effects associated to HCV exposure, even among those cured of the infection. As such, transplanting livers from HCV-infected donors into uninfected recipients requires special approval from governing bodies in the United States and in nearly all countries around the world[120].

HCC as a consequence of DAA treatment

Regarding the plausibility of HCC development in the context of DAA therapy, a risk reduction would be expected as viral clearance reduces morbidity and mortality rates. It should be considered, however, that HCV has a direct carcinogenic potential since some of the HCV-encoded proteins interact with cellular regulatory factors and produce oxidative stress, DNA damage, and deregulation of host cell checkpoints, thus promoting tumorigenesis[121,122]. Because HCV is an RNA virus and its genome does not undergo reverse transcription into DNA, its carcinogenic effects cannot be attributed to its integration into the hepatocyte genome. Lately, the risk of occurrence or recurrence of HCC in HCV patients who received DAA has been debated. IFN-based therapy reports demonstrated that achieving a SVR significantly diminished the risk for HCC[123]. Furthermore, these patients recover liver functionality with a positive impact on long-term disease-free survival[121,124]. On the other hand, initial reports on DAA therapy exposed a potential high risk of HCC occurrence and recurrence after treatment[121,125-127] and hypothesized that the occurrence of HCC would be the result of the emergence and spread of a pre-treatment "orphan" tumor clone that escapes immunological surveillance. The rapid virus elimination following DAA therapy may lead to an imbalance of the immunity that may rebound on immune control of the neoplastic clone[121]. However, the initial results were not conclusive or were even opposed due to the lack of homogeneity in the study design [125]. Recent seminal reports support the notion of a reduced rate for occurrence or recurrence of HCC after SVR obtained after DAA treatment, so the impact of DAAs on HCC risk is nowadays an old tale[93,128,129]. However, the time of DAA therapy initiation in HCC HCV positive patients is still controversial since it seems to condition treatment success[2,125,130,131]. In this sense, the best advice for physicians is to follow approved international or local guidelines and to keep updated to minimize risks or therapeutic failures[2,125,130,131].

Vaccine

The development of a prophylactic HCV vaccine that can contribute to the eradication goal still remains as a pending issue. The diversity of the virus, different behaviors of the virus in animal models or cell cultures, the limited models or individuals to test the vaccines, and the insufficient understanding of protective immunity against HCV are barriers to the development of an effective vaccine. It has been described, both in chimpanzees and humans, that immune system surveillance of primary infection is not necessarily efficient in controlling a recurrent one[132,133]. Therefore, spontaneous HCV immune control does not certainly generate protective immunity, hence diminishing confidence that prophylactic vaccination is possible. Furthermore,

compared to the initial HCV infection, a lower peak and duration of viremia characterized the reinfection in the same individual[113,134,135]. A faster and more effective viral replication control at second exposures indicates an adaptive immune response that may avoid chronic infection even though it cannot prevent reinfection. Therefore, a vaccine that induces T and B cell responses against multiple HCV genotypes and impedes the selection of virus escape mutants is needed[111,113]. While attenuated vaccines by the passage of the virus in non-human primate cell lines could be produced and suppress or inactivate genetic virulence factors, HCV does not replicate at high levels in non-human primate cell lines and no virulence factors have been defined for HCV yet. Therefore, concerns related to the production and the potential risk of attenuated vaccines could limit their utility[113]. In addition, HCV culture strains have adaptive mutations that enhance their ability of *in vitro* replication with an unknown impact on replication in humans. Inactivated whole HCV vaccines were also described; however, the lack of effective processes in the later phases for the purification of HCV represents an obstacle for the development of a complete virus vaccine[136]. On the other hand, there are numerous approaches involving viral antigens as immunogens, namely DNA-vaccines, adenovirus-based strategies, virus-like particles, HCV recombinant antigens conjugates to HBsAg, and HCV peptides in different delivery platforms[113,137-143]. Most of these candidate's vaccines have triggered humoral and cellular immune responses in rodents, and a small subset of them causes immunity in macaques, and fewer candidates in chimpanzees[113,144-148]. Likewise, only a few HCV vaccine developments accomplished the goal of phase I trials in volunteers not at risk for HCV infection[113,149-153]. Given that the partial results of the clinical trials are not completely encouraging, new strategies are required to improve and/or maintain antiviral immunity, and therefore there is a long way to go until a successful HCV vaccine could be used[137].

HCV infection is an example of the success of translational research, as a result, HCV infection is the only chronic viral infection that can be cured, and the hepatic or extrahepatic manifestations are mostly reversible[154]. Many countries are making significant progress in their fight against it, but HCV surveillance is at the base of any effort to control and eliminate the disease, since early diagnosis can prevent health problems that may result from infection and prevent transmission of the virus. The road is long, but with clear objectives the goal can be achieved.

HDV

In 1977, Rizzetto *et al*[155] identified a new antigen in the liver and serum of HBV infected patients who showed more severe hepatitis than their counterparts[155]. This observation led to the discovery of the HDV (also called a satellite virus), an unusual defective virus whose genome consists of a negative single-stranded circular RNA that encodes a single nucleocapsid protein, the delta antigen. The HDV virion, of 36 nm, consists of a ribonucleoprotein core complex and a lipoprotein envelope composed of the three HBV envelope proteins: Small (S-), medium (M-), and large (L-) HBsAg. HBV presence is mandatory for HDV replication, since HBsAg is required for HDV cell entry by NTCP, virion assembly, and export; however, its RNA replication is autonomous. HDV is maintained as episomes in the nucleus of the infected hepatocytes and transcribes the viral RNAs on behalf of the host cell machinery[156].

Clinical, epidemiological, and virological features

Two different scenarios may allow HDV infection: Either HBV and HDV simultaneously infect the host (co-infection) or HDV infection occurs in CHB patients (superinfection). In general, HDV is a highly pathogenic virus associated with more severe forms of acute hepatitis, including fulminant hepatitis. The natural course and outcome of acute hepatitis D differ according to the way infection takes place, whereas only 2% of coinfections evolve to chronicity, superinfection results in chronic infection in over 90% of the cases[157]. Irrespective of the type of infection, the chronic state leads to more severe hepatitis associated with higher rates of HCC and a faster progression to cirrhosis compared with HBV monoinfection, increasing this risk three times among HDV-HBV coinfected patients[157,158]. At least 5% of individuals with chronic HBV are co-infected with HDV, raising the HDV global burden of infection to an estimate of more than 62 million people, nearly 1% of the world's population[2,159]. Despite having a global distribution, HDV has a higher prevalence in Africa (Central and West Africa), Asia (Central and Northern Asia), Pacific Islands, Middle East, Eastern Europe, South America (Amazonian basin), and Greenland[2,160,161].

In addition to host factors, HDV and HBV genotypes influence the course of chronic hepatitis[162]. HDV genome analysis disclosed at least eight distinct HDV genotypes (HDV-1 to -8), with some displaying two to four sub-genotypes. Infection with

genotype 1, the most common one, has been associated with a wide spectrum of disease severity, while other genotypes appear to be more geographically restricted and to be linked with different degrees of disease severity. Infections with either genotype 2 and 4, the most commonly genotypes found in the Far East, generally develop milder forms of liver disease, whereas genotype 3 exclusively found in the Amazon region, has been documented as one of the most aggressive types, associated with severe and fulminant hepatitis outbreaks. HDV-5 is predominant in West Africa, whereas HDV 6, 7, and 8 were isolated in patients from central Africa[157]. Furthermore, HBV genotype could influence HDV infection and replication, being HDV viral loads are lower in patients co-infected with HBV genotype A, whereas co-infection is more frequently seen in genotype F CHB patients[156].

Transmission and diagnosis

HDV and HBV routes of transmission are alike, namely intravenous drug users or exposure to infected blood products and serous body fluids, but HDV mother to infant transmission is rare[162]. HIV infection, intravenous drug users, men who have sex with men, and individuals from areas of high HDV prevalence who are HBV-infected are at risk for co-infection with HDV[2,161]. The HDV antigen is only detectable transiently, therefore the diagnosis is made by measuring anti-HDV antibodies. HDV IgM appears in blood between the first and third weeks after infection and remains positive in the chronic phase with variable levels according to disease activity. HDV IgG is also detectable during active and resolved infection, so this test is useful for the screening of chronic or past HDV infection, while HDV RNA detection is applied to confirm active chronic hepatitis and to supervise therapy response. Anti-HDV IgM and HDV RNA assessment, together with HBV infection acute markers, should be tested to distinguish between acute co-infection HBV/HDV *vs* HDV superinfection [163].

Immune response and pathogenesis

Experimental and clinical studies suggested that HBV is a weak inducer of innate response and has developed strategies to evade innate immune sensing, whereas HDV has shown to activate the IFN pathway *via* melanoma differentiation antigen 5. It has been suggested that both HBV and HDV could inhibit the janus kinase/signal transducer and activator of transcription signaling pathway and hence the response to exogenous IFN. So, the constant activation of the IFN pathway may contribute to chronic viral pathogenesis; however, the implications on liver disease of HDV and HBV and innate immunity interplay remain to be understood. HDV activation of the type-I IFN pathway may promote an increase in the NK cell number, thereby inducing the killing of HBV-specific CD8 T cells by tumor necrosis factor-related apoptosis-inducing ligand-dependent mechanisms, hence worsening HBV pathogenesis in co-infected patients. Additionally, it has been described that HDV proteins affect autophagy by promoting HDV replication, cause oxidative stress, and modulate the transforming growth factor-β and nuclear transcription factor-kappa B signaling pathways. However, most of the studies have been performed in artificial systems that naturally tend to overexpression, so most of them need to be confirmed in actual infectious systems[156,164,165].

HDV pathologic changes are limited to the liver with histopathologic features that are not specific for it, but they tend to be more severe in HDV disease. The hepatocyte injury is typically focal, except in the most severe cases when confluent necrosis occurs, leading to submassive or massive necrosis accompanied by infiltration of inflammatory cells within the collapsed lobules and in the portal areas[157]. Liver biopsy is still of election to achieve an accurate inflammation grading and fibrosis staging since fibrosis noninvasive markers are not reliable in chronic HDV infection. The higher inflammation in HDV compared to HBV monoinfection alters elastography measurement, so the accuracy of transient elastography seems to be reasonable to detect cirrhosis but remains to be validated for grading lesser degrees of fibrosis[1,166-168].

Viral tropism

Several studies have proved the ability of HDV to replicate in a variety of tissues and cells after transfection; moreover, HDV-like viruses have been isolated from other species (birds, snakes). These findings question the hypothesis of an escaped human gene HDV origin and also alludes to the cooperation with other viruses to egress. Furthermore, it has been shown that HDV ribonucleoprotein can be assembled with envelope proteins that come from non-HBV related viruses, raising the question of HDV may also be harbored by other viruses[156].

Treatment

The ability to achieve SVR in the treatment of HDV remains uncertain given the high rates of late relapse. Therefore, HDV remains difficult to treat with the current available therapies. PEG-IFN is the election therapy but the absence of HDV treatment guidelines generate uncertainty concerning protocols. Nucleoside/NAs are ineffective because they do not reduce HBsAg levels, which is required for HDV propagation. However, despite the presence of HDV typically suppressing HBV replication, nucleoside/NA (entecavir or tenofovir) is generally recommended, particularly in patients with cirrhosis, regardless of HBV replication status[1]. Nevertheless, the Hep-Net International Delta Hepatitis Intervention Trial, a large multicenter program, treated patients with PEG-IFN-a-2a and/or adefovir for 48 wk. Six months after treatment completion, 28% of patients who were treated only with interferon continued to have undetectable HDV RNA with no additional benefit compared to those who also received adefovir and showed no response in individuals treated with adefovir alone. In a consecutive study in which patients were treated with PEG-IFN-α with or without tenofovir, only 23% of patients with interferon therapy presented levels of RNA under the detection limit 24 wk after stopping treatment with no extra benefit from the additional use of tenofovir[169]. Therefore, treatment is still the greatest challenge in HDV infection. So far, several promising new therapies have been described, some of which in combination with interferon, may result in sustained clearance of HDV. In this regard, myrcludex and lonafarnib are two promising treatments that are at the most advanced development stages. Myrcludex is an entry inhibitor while lonafarnib prevents HDV secretion, preventing both *de novo* and reinfection cycles. Other therapies are under evaluation in different clinical trials, such as hepcludex which has already been partially approved owing to its safe profile[170,171].

Despite these promising advances, we are in need of treatments achieving permanent HDV RNA suppression since high rates of relapse are associated with current IFN therapies in addition to increased transaminase levels after discontinuation. Interestingly, HDV coinfection prior to liver transplantation reduces the risk of graft reinfection and is associated with better patient survival than HBV-monoinfected patients. However, reinfection with HDV following liver transplantation may still occur, but tends to be aborted if HBV recurrence is also prevented[2,172].

The current knowledge on HDV highlights that the critical points to be addressed in future research must be directed to explain the virus and the immune system interaction linked to the pathogenesis that might allow treatment improvement against chronic liver disease produced by HDV.

HEPATITIS E VIRUS

The hepatitis E virus (HEV) is a causative agent of endemic and epidemic hepatitis worldwide, producing approximately 20 million infections every year, leading to an estimated 3.3 million symptomatic cases[173]. It is a spherical, non-enveloped virus that belongs to the family *Hepeviridae*, genus *Orthohepevirus*, a genus that is divided into four species (A-D)[174]. The strains of species A (Orthohepevirus A) are responsible for hepatitis E in humans. It comprises eight genotypes (HEV-1 to 8) displaying a geographical distribution and different epidemiological patterns. Genotypes that infect humans are 1-4 and 7[174,175].

HEV represents a significant public health challenge in resource-limited settings, mainly from Asia and Africa. In industrialized countries, it has historically been incorrectly regarded as having little clinical relevance[4]. However, in the last years, it has been recognized as an emerging and often undiagnosed disease in developed countries and some places of America, based on increasing reports of non-travel associated sporadic cases and chronic clinical presentations[176].

Epidemiology and transmission: Old and new challenges

Two epidemiological patterns have been observed for HEV. The first one is related to genotypes 1 (HEV-1) and 2 (HEV-2), which infect only humans and are transmitted mainly by the fecal-oral route, through water contaminated with the virus, resulting in frequent sporadic cases and occasional large outbreaks. These genotypes circulate in areas of high endemicity, generally in developing countries (due to poor sanitation) in Asia, the Middle East, North Africa, and some parts of America[175-177]. The second pattern, observed mainly in industrialized countries and some parts of America, is related to the zoonotic transmission of HEV genotypes 3 (HEV-3) and 4 (HEV-4), in

which pigs are considered a viral reservoir, although these viruses have also been detected in other animals, such as wild boar or deer[175,177]. Humans can become infected through direct contact (with many studies showing that farmers have higher levels of HEV antibodies)[178] or by ingestion of raw-undercooked animal meat or derived products, such as sausages or pates, that contain the virus[5]. Shellfish, fruits, and vegetables have also been implicated in viral transmission, probably due to pig slurry contaminating watercourses, which are used for irrigation, or being used as fertilizer[4,5]. HEV-3 has a worldwide distribution, while HEV-4 is restricted to Asia and Europe[177]. Interestingly, genotype 7 (HEV-7) has only been described in the Middle East and Dubai, from sporadic human cases and camels[174].

Since HEV is transmitted by the fecal-oral route (involving contaminated waters) and also as a zoonosis (having animal reservoirs), many studies in the last years have focused on HEV detection in environmental and food matrices as sources of HEV infection[177]. HEV has been detected in many environmental matrices, such as sewage, recreational waters (river, creek, dam), and tap waters, showing fecal contamination of the environment[179,180]. Viral presence in sewage represents an indicator of viral excretion of a given population, so it is useful for monitoring HEV circulation [181]. Water resources that are contaminated with wastewater are the main origin for the dissemination of enteric viruses and, in consequence, they could represent a viral reservoir with a dramatic impact on the population's health[179].

HEV food contamination that is not derived from pork is another route that is currently being studied, such as shellfish, fruits, vegetables, or milk, which have been postulated to be possible sources of infections, particularly in places where sporadic cases without an epidemiological link occur[5]. Many new lines of study are focused on the research of HEV in foods, methodologies for its detection in food matrices, and food outbreaks. The knowledge of these sources of infection will allow for improvements in the prevention of HEV infection.

Additionally, vertical transmission from mother to child[182] and transmission through blood transfusion[183] have also been described, but as less frequent routes. However, the transfusion route is currently becoming more relevant since an increasing number of cases are being reported in Europe and Asia[176]. This is particularly important for immunosuppressed populations since these patients could develop chronic infections and are commonly subjected to blood transfusions. Asymptomatic carriers of HEV could play a possible role as human viral reservoirs, and the virus can be transmitted during the donation, when the volunteer donates blood prior to the onset of the acute stage of hepatitis E[176]. In response to the threat posed by HEV to transfusion safety, many European countries have implemented screening for HEV-RNA in blood products, and many others are considering to do so [183]. However, in the rest of the world, there is still a lack of knowledge about this route of transmission.

In recent years, a few cases of acute and chronic human hepatitis E attributed, for the first time, to the HEV-C (*Orthohepevirus C*) species were reported in many parts of the world. Until now, this virus had only been detected in rats and ferrets (known as rat-HEV), and belongs to the genus *Orthohepevirus*, as well as the human-infecting HEV-A, although they are very divergent[4,184]. HEV-C genotype 1 was identified in both immunocompetent and immunocompromised patients who displayed acute and chronic infection as described in a recent large prospective study in Hong Kong, positioning this virus as a new etiological agent of hepatitis E. It is important to mention, as observed in one case, that the pre-existing HEV antibodies did not protect against HEV-C genotype 1. Also, routine hepatitis E diagnostic tests may overlook HEV-C infection[184]. Therefore, this is a new challenge in the field of viral hepatitis and specifically in understanding HEV epidemiology.

Clinical features

In most cases the infection produces an acute self-limited illness with a variety of clinical manifestations, ranging from asymptomatic course to acute liver failure, resulting in fatality rates of 0.2%-4%. The most common symptoms are abdominal pain, nausea, vomiting, anorexia, fever, and jaundice[182]. The course of the disease could be more severe in pregnant women infected with HEV-1, with high maternal, fetal, and neonatal morbidity and mortality rates, as high as 25%[182]. In turn, it has been described in individuals who have chronic liver disease that the mortality rate increases when infected by HEV[174]. Chronic HEV infections have been identified among immunocompromised persons infected with HEV-3 or HEV-4, including patients receiving cancer chemotherapy, recipients of organ transplant, and HIV-infected persons. In these cases, HEV-RNA had been detected in serum and/or stool samples for at least 6 mo[175,177]. Chronic hepatitis E seems to be an increasing

problem, particularly in Europe, where areas with high chronicity rates have been identified.

Hepatitis E also shows a spectrum of serious complications and extrahepatic manifestations, which are being increasingly recognized[174]. Some of them include acute or chronic liver failure, neurological disorders, pancreatitis, renal injury, cryoglobulinemia, hematological disorders, and thyroiditis[174,175,177]. The mechanisms of HEV-associated extrahepatic injuries are not fully understood yet and represent a challenge for the study of hepatitis E and its management.

Diagnosis

Diagnosis of hepatitis E infection can be carried out using direct techniques, which allow for the detection of the viral antigens and nucleic acid, as well as by the detection of IgG and IgM HEV-specific antibodies, although it may require a combination of both, molecular and serological assays, to confirm infection and for monitoring the treatment in chronically infected patients[185]. Laboratory diagnostic techniques for HEV detection vary in their specificity and sensitivity, something important to consider when using any of them, and to make comparisons. Currently, the gold standard test is the PCR for HEV-RNA amplification[185]. In the case of acute hepatitis E, a differential diagnosis should be performed to exclude other viral hepatitis and other causes (autoimmune, toxic, *etc.*) of liver disease. HEV-RNA detection can be carried out in serum samples (although the viremic period is short) as well as in stool samples, in which virions are shed for a longer period of time[4]. HEV antigen can also be performed, using double-antibody sandwich enzyme immunoassay techniques, which can be detected in serum, feces, or urine[185], although it is not extensively used. Acute hepatitis can also be diagnosed by IgM anti-HEV detection[173]. It is worth mentioning that the time of diagnosis and sample extraction is crucial. HEV is not generally taken into account in an initial assessment of a sick individual, due to still being regarded as an "emerging" disease, and many clinicians have limited knowledge of the disease[4]. This delay in sampling could lead to false negative results for viral RNA detection. In these cases, specific IgM testing is useful. For chronic infections, diagnosis is performed by detecting the presence of HEV-RNA by RT-PCR (and/or its variants Nested-PCR and RT-PCR) in blood for more than 6 mo[174]. The titer of antibodies against HEV may be lower in these patients, as well as in those immunosuppressed, so detection of HEV IgM and IgG should be interpreted with caution[185]. Although viral genotyping is not routinely performed, its determination is important in order to understand the clinical and epidemiological pattern (especially in risk patients, as immunosuppressed individuals, pregnant women, *etc.*), as well as for viral surveillance and to monitor the introduction of new genotypes/strains in a given region.

Antiviral treatment and vaccine

Antiviral therapy is not usually required in acute HEV infection since the virus is spontaneously cleared. However, treatment with ribavirin may be considered in cases of severe acute hepatitis E or acute-on-chronic liver failure[174].

In the case of chronic infections, ribavirin monotherapy for 3 mo is recommended. Decreasing levels of immunosuppression at diagnosis of chronic HEV infection is also advisable in solid organ transplant recipients. After 3 mo, HEV-RNA should be assessed in stool and serum samples. If RNA is undetectable, European Association for the Study of the Liver suggests stopping ribavirin therapy. If RNA replication persists, therapy with ribavirin should be continued for an additional 3 mo (6 mo course of ribavirin monotherapy in total). In the case of liver transplant recipients with lack of response to ribavirin, PEG-IFN therapy for 3 mo could be considered[174,186].

Even though many HEV vaccines have been developed worldwide, only one has been licensed in China (Helicon®). This vaccine is based on a recombinant HEV peptide derived from genotype 1, corresponding to a fragment of the open reading frame 2, which encodes the capsid protein of HEV. It is recommended to be used in individuals aged > 16 years and at high risk of HEV infection (food handlers, animal husbandry, soldiers, women of childbearing age, travelers to endemic areas, *etc.*). However, very little is known about many aspects of this vaccine, which limits its use, such as the efficacy (it has only been proved to prevent symptomatic hepatitis E due to genotype 4), immunogenicity and safety, especially in specific populations, like pregnant women, transplant patients and subjects with chronic liver disease[187].

The foregoing highlights new challenges regarding hepatitis E worldwide, showing that further research about epidemiological, clinical, and virological aspects are needed to understand better the different HEV scenarios and implications around the world.

CONCLUSION

In this review, we summarized the most relevant topics that are being analyzed or that have recently arisen in the setting of viral hepatitis. Although in recent years significant progress has been made in the knowledge of viral hepatitis, there are still many aspects to be resolved. It is necessary to continue working on improving diagnosis to maintain a constant and continuous epidemiological follow-up of infected populations, expand knowledge on the mechanisms of pathogenesis of each virus, improve treatment, and develop or improve the efficiency of vaccines. It is important to understand that strategies must be both local and global, as this represents that most successful path for viral hepatitis to cease being a major public health problem.

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REVIEW

Biomarkers in the diagnosis of pancreatic cancer: Are we closer to finding the golden ticket?

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Abstract

Pancreatic cancer (PC) is a leading cause of cancer related mortality on a global scale. The disease itself is associated with a dismal prognosis, partly due to its silent nature resulting in patients presenting with advanced disease at the time of diagnosis. To combat this, there has been an explosion in the last decade of potential candidate biomarkers in the research setting in the hope that a diagnostic biomarker may provide a glimmer of hope in what is otherwise quite a substantial clinical dilemma. Currently, serum carbohydrate antigen 19-9 is utilized in the diagnostic work-up of patients diagnosed with PC however this biomarker lacks the sensitivity and specificity associated with a gold-standard marker. In the search for a biomarker that is both sensitive and specific for the diagnosis of PC, there has been a paradigm shift towards a focus on liquid biopsy and the use of diagnostic panels which has subsequently proved to have efficacy in the diagnosis of PC. Currently, promising developments in the field of early detection on PC using diagnostic biomarkers include the detection of microRNA (miRNA) in serum and circulating tumour cells. Both these modalities, although in their infancy and yet to be widely accepted into routine clinical practice, possess merit in the early detection of PC. We reviewed over 300 biomarkers with the aim to provide an in-depth summary of the current state-of-play regarding diagnostic biomarkers in PC (serum, urinary, salivary, faecal, pancreatic juice and biliary fluid).

Key Words: Pancreatic cancer; Cancer; Biomarkers; Diagnostic; Review

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Core Tip: Circulating biomarkers are an attractive method for pancreatic cancer (PC) diagnosis. Over 300 biomarkers are presented in this review, however no gold standard biomarker exists. While carbohydrate antigen 19-9 possesses modest sensitivity in PC diagnosis, a lack of specificity is a limitation for its use. More recent studies have shifted towards the concept of a liquid biopsy along with measuring expression of RNA based markers in different mediums. Panels comprising multiple candidate biomarkers have emerged, demonstrating modest diagnostic value. Further studies are required to validate these findings, along with assessment in an asymptomatic population to determine their value in screening.

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INTRODUCTION

Pancreatic cancer (PC), most recently declared as a medical emergency by the United European Gastroenterology in a position paper, is a leading cause of cancer related mortality on a global scale, being the 12th most common cancer diagnosis, and the seventh leading cause of cancer related death[1-3]. The mortality associated with PC is significant compared to its solid organ tumor counterparts, accounting for approximately 4% of cancer related deaths with a Mortality/Incidence ratio of 98%, and has a dismal 5-year survival rate of approximately 9% which has only incrementally improved over the past forty years due to improvements in neoadjuvant and adjuvant therapeutic options[3-5]. This poor prognosis is attributed to patients being diagnosed with advanced disease at the time of presentation and the relatively silent nature of the disease[6]. It is estimated that, at the time of diagnosis 80%-90% of patients have unresectable disease[7]. It is postulated that diagnosis at an earlier stage would increase the 5-year survival rate as this would allow for curative resection along with adjuvant chemotherapy[8,9].

Due to the overwhelming number of patients having unresectable disease at the time of diagnosis there has been an emphasis on the identification of novel diagnostic modalities or biomarkers that can assist clinicians in detecting PC at an early stage. Currently there is no defined PC screening strategy for the general population that is comparable to screening colonoscopies for colorectal cancer (CRC) and the programs that exist are only limited to high risk patients (familial PC and hereditary PC syndromes) which represent only 5%-10% of all PC patients[10-12].

The goal of early detection of PC in otherwise asymptomatic patients is optimistic however so far impractical due to low incidence of PC in the general population, where even with a screening assay with a high specificity, implementing a screening program might result in increased levels of anxiety in the screened population with the potential for false positive results[13]. Further to this, the vast majority of studies have assessed the utility of diagnostic biomarkers in patients with symptomatic disease, rather than as a surveillance or screening biomarker in the general population.

A biomarker is defined as 'any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease'. Currently carbohydrate antigen 19-9 (CA19-9) is regarded as the best serological biomarker available so far in the diagnosis of PC, however the majority of studies endorsing the use of CA19-9 as a complementary test in the diagnosis of PC acknowledge it is not specific or sensitive enough to be used for screening[14,15]. A number of other biomarkers have been proposed and these will be reviewed here[16]. Variation exists in the biomarker domain, with studies utilizing serum, biliary fluid, pancreatic juice, urine, faeces and pancreatic cystic fluid for analysis of potential agents to determine their worth as a malignancy biomarker, however these methods of assessment vary in their invasiveness, sensitivity and specificity[17-20].

Due to the currently rapidly evolving landscape of potential biomarkers for early diagnosis of PC and the apparent lack of a gold standard diagnostic assay in the general population, the aim of this review is to provide a comprehensive update on the current diagnostic biomarkers implicated in PC with over 300 biomarkers

reviewed here.

SEROLOGICAL BIOMARKERS OF PC

Serum has been the most utilized modality for specimen collection for biomarker analysis, and it is the preferred specimen for analysis due to simplicity of collection and low risk, however it has limitations, particularly the potential for dilution of candidate tumour markers and the potential for these markers to be obscured by other serum proteins that exist within samples[21].

Glycolipids and proteins

CA19-9: CA19-9 is a tetrasaccharide expressed on the surface of cancer cells. It is the most well-known serological biomarker used in PC diagnosis, and was initially described in 1979 as a tumor antigen recognised by the monoclonal antibody NS19-9 in the case of CRC[22,23]. CA19-9 is not specific for PC alone, and has been implicated in colon, gastric and biliary tract cancer[24-26]. CA19-9 has only been reported to be elevated in only 80% of all PC patients, and has been used in monitoring disease progress or responsiveness to treatment[27,28]. CA19-9 has also been demonstrated to be elevated in benign conditions such as chronic pancreatitis (CP), biliary obstruction and cholangitis highlighting a lack of specificity[29,30]. In addition to this, CA19-9 is related to the Lewis blood group antigens and only those patients who belong to the Le (α-β+) or Le (α + β-) blood groups will express the antigen, its sensitivity in the diagnosis of PC is questionable as 10% of the population have a Le (α-β-) phenotype which lacks the enzyme 1,4-fucosyl transferase that is essential for the production of CA19-9[31,32].

Only a scarce number of studies have evaluated serum CA19-9 Levels in the general, asymptomatic population as a screening modality for PC. These studies were conducted in Japanese, Korean and Taiwanese populations and reported a low positive predictive value (PPV) of serum CA19-9 in the diagnosis of PC in a screening setting[33-35].

A recent meta-analysis assessing the diagnostic value of CA19-9 in PC compared to carcinoembryonic antigen (CEA) reported a summary sensitivity of 0.80 in the diagnosis of PC, along with a summary specificity of 0.75 and area under the curve (AUC) of 0.84[36].

To improve the diagnostic performance of CA19-9, it has been combined with a number of other biomarkers in the research setting[37,38]. This has translated to improved diagnostic value. Of note, sialylated tumor-related antigen, including sialyl-Lewis A glycan isomers, has recently been demonstrated to be superior to CA19-9 when used in isolation, as well as improving the sensitivity and specificity when used in combination with CA19-9[39-41] (Table 1).

CEA: CEA is a foetal glycoprotein that is not usually produced in large quantities after birth. Aside from its role in the surveillance and prognosis of CRC, CEA has also been implicated in ovarian, cervical, lung and breast cancer[42]. A number of studies have investigated the diagnostic value of CEA for PC, however the results reported are inconsistent throughout the literature.

The predictive value of CEA in the diagnosis and prognosis of PC has been recently evaluated in a relatively small systematic review and meta-analysis published by Meng *et al*[43] in 2017. Through the analysis of 19 studies including 3650 participants, a CEA-based panel was deemed to have greater diagnostic accuracy compared to CEA or CA19-9 alone with an AUC and *Q* value of 0.90 and 0.84 respectively, however the sensitivity of the panels demonstrated no advantage over CA19-9 or CEA when utilized in isolation[43]. A meta-analysis conducted in 2018 comparing CA19-9 to CEA included 13 studies with 4537 participants and 1277 patients diagnosed with PC[36]. This study demonstrated a superior sensitivity of CA19-9 compared to CEA (ratio of sensitivity = 1.54), along with a superior AUC (ratio of AUC = 1.24). A recommendation was made that both markers should be utilized for early diagnosis of PC due to their convenient, efficient and non-invasive properties.

CA125: CA125 is a high-molecular-weight mucin-like glycoprotein that has been associated with ovarian cancer, CRC and cholangiocarcinoma[44-46]. The role of CA125 in PC has only been established in the past decade with small studies demonstrating its superiority to CA19-9 in predicting resectability of PC, along with correlating with metastasis-associated disease burden[47,48]. There is unique clinical utility for CA125 given that serum levels do not correlate with serum bilirubin levels

Table 1 Serum protein biomarkers implicated in the diagnosis of pancreatic cancer

Class	Candidate marker
Glycolipids and proteins	CA19-9[27,28,33-38,144,160,182,187,213,221], sTRA[39-41], CEA[43], CA125[47,48,50], CA242[55,53], Osteonectin[57], Osteopontin[58-61], DUPAN-2[65-70], LAMC2[73-75], ULBP2[78-80], sCD40L[82], LRG1[84], C4BPA[86], Cofilin-1[88], sgC1qR[91], Trypsinogen-2[92,93], DKK1[96], THBS-2[99-102], THBS-1[103], AGR2[108], REG1A[108], REGIII[108], REG1β[111], REG4[114-117], SYCN[108], LOXL2[108], PARK7/DJ-1[126], TTR[129,130], TTF1[134], TTF2[134], TTF3[134], GPNMB[138], PRX-1[139], TFPI[141], TIMP-1[144], MMP-9[144], IGFBP-1[146], IGFBP-2[147-149], IGFBP-3[147,149], MSLN[148,154], C5[152], MMP-7[155-157], cathepsin-D[156], MMP-12[157], OPG[160], Kisspeptin[165], Galectin[171], MUC16[48,182], MUC5AC[37,182], PAM4[187], HSP27[190,191], CAM17.1[192,193], Fuc-Hpt[194], SAA [196], APN/CD13[200], M2-PK[203,204], APOA2[206-208], APOC1[209], APOC2[210], APOE[211-212], ITIH[213], APOA1[213], APOL1 [213]
Growth factors	TGF-B[215], VEGF[217], FGF-10/KGF-2[138], PDGF[220], TSGF[221]
Cytokines and chemokines	IP-10[220], IL-6[220,230-232], MIC-1/GDF15[227,228], IL-11[229], YKL-40[232,233], IL-8[230,234,235,237,241], IL-10[214], IL-1β[214], OSM [138], TNF-α[240-244], M-CSF[214], CXCL11[138], SCF[138,247-248], Eotaxin[250], HGF[250], MCP-1[250], CXCL10[250]
Adhesion molecules	CEACAM1[253,254], ICAM-1[160,262-263]

CEA: Carcinoembryonic antigen; TTF: Thyroid transcription factor; sTRA: Sialylated tumor-related antigen; IL: Interleukin.

and it is not significantly altered in the case of patients who are jaundiced[49].

A recent meta-analysis comprising eight studies with 1235 participants demonstrated a pooled sensitivity of 59% and specificity of 78% for CA125 in the diagnosis of PC, while the AUC and Q-value of the CA125-based diagnostic panel were 0.89 and 0.82 respectively[50]. This panel was deemed to be superior to CA125 or CA19-9 when used in isolation. Although this demonstrated a favourable result for the use of a CA125-based diagnostic panel going forward, the meta-analysis was limited by its size and heterogeneity between studies.

CA242: CA242 is a sialic acid-containing carbohydrate antigen which has been reported to have a high correlation with CA19-9 in patients diagnosed with PC[51-53]. Serum CA242 has also been demonstrated to be highest in patients diagnosed with PC compared to other solid organ malignancies, such as cervical cancer or oesophageal cancer[54].

In a 2015 meta-analysis comprising 21 studies and 3497 participants, CA242 was evaluated in conjunction with CA19-9 and CEA in diagnosing PC[55]. CA242 pooled sensitivity for detection of PC was 67.8%, with a subsequent pooled specificity of 83.0%. When combined with CA19-9, a sensitivity of 90.0% was achieved. More recently, a biomarker panel of CA19-9, serum periostin (POSTN) and CA242 was able to discriminate early stage PC from controls with an AUC of 0.98, along with benign conditions (AUC = 0.90)[53]. When utilized in isolation however, receiver operating characteristic (ROC) curve analysis returned an inferior result for CA242 in comparison to CA19-9 in distinguishing early stage PC from healthy controls.

Osteonectin: Osteonectin is a glycoprotein that has been previously demonstrated to have a key function in PC through promoting invasion and metastasis[56]. There is limited data on the use of Osteonectin in the diagnosis of PC, with a small prospective study reporting significantly elevated serum levels in those diagnosed with PC compared to controls, and a plasma level of > 100.18 ng/mL on ROC curve analysis resulting in an AUC of 86% for predicting PC[57].

Osteopontin: Osteopontin (OPN), a protein associated with the extracellular matrix (ECM), has been previously reported to be upregulated in PC preoperative serum, where when elevated it was found to have a sensitivity and specificity of 80% and 97% [58]. More recently serum levels of OPN and tissue inhibitor of metalloproteinase 1 (TIMP-1) were able to distinguish PC from CP and healthy controls. Additionally, when combined with CA19-9, diagnostic accuracy improved than compared to when used in isolation[59].

A meta-analysis published in 2014 demonstrated that the serum OPN levels in patients with PC was significantly greater compared to controls[60]. More recently, a pilot study published in 2016 identified that levels of OPN were higher in patients with PC compared to those with CP and control subjects, further affirming its potential role as a diagnostic biomarker in PC[61].

Duke pancreatic monoclonal antigen type 2: Duke pancreatic monoclonal antigen type 2 (DUPAN-2) is the precursor for CA19-9 has been reported to be elevated in patients with PC who are negative for the Lewis blood group phenotype highlighting an advantage over the conventional biomarker CA19-9[62-64]. There is minimal literature evaluating serum DUPAN-2 in the diagnosis of PC and the sensitivity of the biomarker in diagnosing PC is less than desirable, with its use shifting from diagnosis to prognosis more recently[65-70].

Laminin γ2: Laminin γ2 (LAMC2), an ECM glycoprotein, has been previously demonstrated to be inversely related to overall patient survival in patients with PC and over-expression has been proposed as a poor prognostic factor in patients diagnosed with PC[71,72]. Its value as a diagnostic biomarker has been assessed in a number of studies where when used in isolation and in conjunction with CA125 and CA19-9 in a panel, LAMC2 has demonstrated efficacy in PC diagnosis[73-75].

UL16 binding protein 2: UL16 binding protein 2 (ULBP2) is an NKG2D ligand present on NK cells that has been implicated in tumorigenesis[76,77]. Initially identified in 2011, ULBP2 was found to be elevated in PC patients compared to healthy controls [78]. ULBP2 has been utilized in combination with MIC-1, where it was reported to be significantly elevated in the serum of patients with PC compared to controls[79]. This elevation of ULBP2 in the sera of patients with PC was further validated in 2017 where in a small single centre study, serum levels of ULBP2, dickkopf-1 (DKK1) and CA19-9 were all significantly elevated in those diagnosed with PC compared to those with benign pancreatic disease and controls[80]. There is very little published with regard to the role of ULBP2 in the diagnosis of PC, with more recent data highlighting a potential role as a predictor of poor prognosis[81].

Soluble CD40 ligand: Soluble CD40 ligand (sCD40L) was first evaluated as a diagnostic and prognostic marker for PC in a study in 2014, where serum levels were significantly elevated in PC patients compared to controls[82]. Considering a lack of validation and small sample size, its routine clinical use is not recommended.

Leucine-rich a2-glycoprotein-1: Leucine-rich a2-glycoprotein-1 (LRG1) is an inflammatory protein present in human sera[83]. Although it was able to distinguish between patients with PC, CP or healthy controls, however the authors were not able to demonstrate effectiveness for LRG-1 as an early diagnostic marker[84].

C4b-binding protein a-chain: C4b-binding protein a-chain (C4BPA) is a serum protein implicated in B cell proliferation and CD40 activation which can reverse immune suppression and stimulate anti-tumour T cell responses[85]. It was demonstrated in a single study to be significantly elevated in patients with PC compared to healthy controls, with a subsequent AUC of 0.860 which was superior to CA19-9[86].

Cofilin-1: Cofilin-1 belongs to a family of proteins known as the actin depolymerizing factor/cofilin family, and has been implicated in chemotaxis, cell migration and tumor cell invasion[87]. There is minimal literature describing the role of cofilin-1 as a diagnostic biomarker of PC, with a single study in 2017 measuring the immune complex levels of cofilin-1 in sera and reporting that levels were significantly elevated in those diagnosed with PC compared to healthy controls and those with CP[88].

Soluble gC1qR: Soluble gC1qR (sgC1qR) is a multifunctional cellular protein which has previously been implicated in inflammation and malignancy[89,90]. With regard to PC, only a single small study has assessed its role as a circulating diagnostic biomarker, where it was demonstrated to be significantly increased in those diagnosed with metastatic PC compared to controls[91].

Serum trypsinogen-2: Serum trypsinogen-2 evaluation as a diagnostic biomarker is limited in the literature. A small study performed in 1996 demonstrated that high levels of serum trypsinogen-2 were present in those with BTC and PC, while also being elevated in benign obstructive disease highlighting a lack of sensitivity associated with the marker[92]. Another small single centre study showed the levels in those with PC and CP were significantly elevated compared to controls[93].

DKK1: DKK1 is a soluble inhibitor of Wnt/B-catenin signalling and has been demonstrated to be over-expressed in a number of solid organ malignancies[94,95]. DKK1 has been previously reported to be superior to CA19-9 on ROC curve analysis in differentiating patients with PC compared to controls with an AUC of 0.919 compared to 0.853 [96], while a more recent review highlights its potential as a target for cancer immuno-

therapy rather than diagnosis[97].

Thrombospondin-2 and thrombospondin-1: Thrombospondin-2 (THBS2) is a glycoprotein that mediates cell-to-cell and cell-to-matrix interactions which has previously been implicated in malignancy, particularly CRC[98]. When utilized with CA19-9, it can boost detection of PC in high-risk populations which has been more recently affirmed[99-101]. Le Large *et al*[102] reported an AUC of 0.952 for THBS2 and CA19-9 in discriminating patients with cancer compared to healthy donors, however there was no difference in plasma THBS2 expression between patients with PC and distal cholangiocarcinoma highlighting a potential diagnostic dilemma and a lack of specificity associated with the assay[102].

Serum THBS1 has been demonstrated to significantly decrease up to 24 mo prior to the diagnosis of PC and when used in combination with CA19-9, an AUC of 0.86 was achieved significantly outperforming both markers utilized in isolation[103].

Anterior gradient homolog 2 protein: Anterior gradient homolog 2 protein (AGR2) is a protein that has been previously identified as having a crucial role in embryogenesis. It is found in the endoplasmic reticulum and on the cell surface, and is expressed by multiple solid organ malignancies[104,105]. It has been previously implicated in the initiation of PC and is expressed in premalignant lesions of the pancreas[106,107]. As a diagnostic biomarker in PC, only a handful of studies exist reporting its elevation in PC compared to controls, with utilisation in a diagnostic assay with CA19-9 and REG1β resulting in modest diagnostic accuracy[108].

Regenerating protein family: REG1β, a member of the regenerating (REG) islet-derived family of proteins, which is present in pancreatic acinar cells, and subsequently is implicated in the regeneration of pancreatic islets[109]. REG family members have also been implicated in PC[110]. REG islet-derived 1 alpha (REG1A) and REGIII were initially demonstrated to be elevated in plasma in murine PC models, while REG1β was first studied in 2013 and was demonstrated to be significantly elevated in PC serum compared to healthy participants and those with benign disease [108,111].

REG4 is also over-expressed in a number of solid organ malignancies, including those of the gastrointestinal tract[112,113]. It acts an antiapoptotic factor through the Akt signalling pathway and has been demonstrated to be elevated in the serum of patient with PC compared to controls[114,115]. Serum REG4 has been reported to be superior to CA19-9 on AUC analysis, however there is inconsistencies in both sensitivity and specificity between studies[116,117].

Syncollin: Usually expressed in pancreatic acinar granules on the luminal side of the granular membrane, syncollin (SYCN) acts to concentrate and mature zymogens, while also regulating exocytosis and has previously been identified in the pancreatic juice of patients diagnosed with PC[118-120]. Initially evaluated in humans in 2013, SYCN was found to be significantly elevated in the serum of patients with PC compared to health controls and those with benign disease. In addition to this, it was also able to identify patients with PC in which serum CA19-9 was normal suggesting superior sensitivity. When combined with the serum biomarker REG1β and CA19-9, it was demonstrated to have an average AUC of 0.895 when discriminating patients with PC compared to healthy controls[108]. Although there is a lack of data to determine whether the findings of the aforementioned studies are generalisable, SYCN does display merit in terms of its sensitivity in patients diagnosed with PC compared to CA19-9.

Lysyl oxidase-like 2: Lysyl oxidase-like 2 (LOXL2) is a member of the lysyl oxidase (LOX) family of secreted, copper-dependent amine oxidases which have been implicated in malignancy due to their ability to promote epithelial-mesenchymal transition[121,122]. Additionally, its expression presents poorer overall survival and worse clinicopathological parameters irrespective of malignancy[123]. LOXL2 has been reported to be elevated in serum of patients with PC compared to controls, however was inferior to CA19-9 and its general ability to distinguish PC from controls was not deemed to be significant[108].

PARK7/DJ-1: DJ-1 is a multifunctional protein which has been implicated in Parkinson's disease, however is also an oncogene that has been demonstrated to be over-expressed in a number of solid organ malignancies[124,125]. DJ-1 was first evaluated in 47 patients with PC in 2011 and shown to be elevated in patients with PC compared to those with CP and controls, with an AUC superior to CA19-9 (0.6647)

[126]. Further studies are warranted to determine whether the results of this study can be replicated.

Transthyretin: Transthyretin (TTR) is the major carrier for the hormones thyroxin and tri-iodothyronine, and has been previously demonstrated to be elevated in patients with endocrine tumours but decreased in solid organ malignancies including epithelial ovarian carcinoma[127,128]. Studies are heterogenous, one study showing serum TTR level decreased by at least 2-fold when compared to control participants and other showing TTR is elevated in patients diagnosed with PC[129,130].

Trefoil factors: Trefoil factors (TFFs) are small, secretory mucin-associated proteins which are involved in the protection of epithelial cells, however an oncogenic role has been noted particularly in the case of gastric cancer[131-133]. In 2019 a small study demonstrated significant elevation of TFF1 and TFF2 in early PC compared to benign controls and CP patients. In addition to this, when combined with CA19-9, the panel of TFF (TFF1, TFF2 and TFF3) resulted in an AUC of 0.93 in discriminating early PC from benign controls[134].

Osteoactivin/glycoprotein nonmetastatic melanoma protein B: Glycoprotein nonmetastatic melanoma protein B (GPNMB) is a type 1 transmembrane protein which has been described as a promoter of metastasis and cellular invasion in malignancy[135-137]. A single study analyzed pre-treatment sera of patients with PC compared to controls and demonstrated modest diagnostic accuracy for PC[138].

Peroxiredoxin-1: Described as an important protector against redox damage, peroxiredoxin-1 (PRX-1) has also been implicated in PC where in the serum of patients it was significantly elevated compared to healthy controls and correlated with aggressive clinicopathological parameters. When combined with CA19-9, the AUC was significantly higher that PRX-1 when utilized in isolation[139].

Tissue factor pathway inhibitor: Tissue factor pathway inhibitor (TFPI) is a plasma Kunitz-type serine proteinase inhibitor which controls coagulation initiation, while also being implicated in malignancy[140]. An isolated study has assessed the role of TFPI in PC, where when utilized in combination with tenascin C and CA19-9 in a biomarker panel, it was demonstrated to improve the diagnostic performance of CA19-9 in discriminating early-stage cancer from healthy controls[141].

TIMP-1: TIMP-1 possesses an inhibitory effect on most MMPs along with playing a role in the regulation of cell proliferation and apoptosis[142,143]. TIMP-1 has a sensitivity of 47.1%, specificity of 69.2% and AUC of 0.64 which, in conjunction with matrix metalloproteinase-9 (MMP-9), were both deemed inferior to CA19-9 as a marker for detecting PC[144].

Insulin-like growth factor binding protein: Insulin-like growth factor binding protein 1 (IGFBP-1) is a downstream target of insulin and inhibits IGF-1 activity[145]. Wolpin *et al*[146] demonstrated that low plasma levels of IGFBP-1 predicted an increased risk of PC in a nested case-control study. In a pilot 2016 study IGFBP-2 and IGFBP-3 were shown to be able to discriminate PC patients with early stage disease from healthy controls, along with being superior to CA19-9 when utilized in combination[147]. Kendrick *et al*[148] showed that IGFBP2 and mesothelin (MSLN) were weak diagnostic classifiers individually but their utilization in a diagnostic biomarker panel was recommended. Additionally, in the case of premalignant lesions, Kim *et al*[149] reported that a biomarker panel of six candidate proteins including IGFBP-2 and IGFBP-3 had high discriminatory power in distinguishing intraductal papillary mucinous neoplasm (IPMN) and controls.

Complement component 5: Component 5 (C5) is a complement protein, which when cleaved into two fragments, C5a and C5b, is implicated in the formation of the membrane attack complex (MAC), a structure that is vital in the innate immune system[150,151]. Wingren *et al*[152] reported that C5 was differentially overexpressed, along with a number of inflammatory and growth factors in the serum of patients with PC compared to normal controls subjects.

MSLN: Initially evaluated in 2009, circulating MSLN was described as a useful biomarker for PC where it was detected in 73 of the 74 patients with PC[153]. However more recently, serum MSLN was found to be a weak diagnostic classifier of PC[148]. This supports the findings of Sharon *et al*[154] who identified that serum MSLN and megakaryocyte potentiating factor did not differ significantly between cohorts

diagnosed with PC, biliary carcinoma, benign pancreatic conditions, healthy controls and benign non-pancreatic conditions, and as such was concluded that it was not useful as a biomarker for the assessment of malignancy.

MMP: In a small study Kuhlmann *et al*[155] reported a 100% positive predictive value when MMP-7 was combined with CA19-9 in patients with periampullary carcinoma. MMP-7 has also been utilized in a panel comprising CA19-9, cathepsin D with an impressive AUC of 0.900 for discriminating patients with PC from normal healthy controls[156]. Kahlert *et al*[157] also reported that serum MMP-7 and MMP-12 were strong classifiers for the diagnosis of patients with PC compared to healthy controls.

Osteoprotegerin: Osteoprotegerin (OPG) is a member of the tumor necrosis factor (TNF) receptor superfamily and is mainly associated with regulation of bone turnover that has also been implicated in malignancy[158,159]. It has been previously combined in a biomarker panel with intercellular adhesion molecule 1 (ICAM-1) and CA19-9 and was able to discriminate PC patients from healthy controls with a sensitivity and specificity of 78% and 94% respectively[160]. This study contrasts with the findings of Nolen *et al*[161] where when combined in a panel with CA19-9 and OPN, OPG was not effective in predicting PC in prospectively collected serum samples in a large screening cohort.

Kisspeptin: Kisspeptin, initially implicated in melanoma, has been demonstrated to be expressed physiologically in a number of different tissues, suggesting it possesses antitumoral properties[162-164]. Recently, in a cohort of 128 patients with PC, serum levels of Kisspeptin were elevated in those with PC compared to healthy controls and ROC curve analysis demonstrated an AUC of 0.797 in discriminating PC from healthy controls, however it was deemed inferior to CA19-9[165].

Galectin-3: Galectin-3 is a member of the β-galactoside-binding protein family which has been previously demonstrated to be associated with a number of solid organ malignancies, including those of the gastrointestinal tract[166-169]. It has been reported to be over-expressed in PC tissue specimens and elevated in the serum of patients with PC[170]. Yi *et al*[171] further built upon this finding in a prospective screening study, where in 1850 healthy participants a single case of PC was diagnosed in a patient with elevated serum levels, a lack of specificity cited as a barrier to implementation.

Mucins: Mucins are a family of glycoproteins that serve a number of functions, and line the surface of epithelial cells in the gastrointestinal tract[172,173]. In normal pancreatic tissue, a number of mucins are expressed, these being MUC1, MUC5B, MUC6, MUC11, MUC12, MUC17, MUC20 and MUC21, while other members of the mucin family are usually undetectable[173-179]. Mucins have previously been demonstrated to have a role in PC in promoting metastasis, chemoresistance and tumorigenicity, while a recent meta-analysis identified MUC1, MUC4, MUC5AC and MUC16 as key biomarkers in the diagnosis of PC[180,181]. On peripheral blood sampling, MUC16/CA125 Levels have been previously demonstrated to be strongly associated with metastatic disease[48]. Serum MUC5AC has also been reported to have efficacy in differentiating resectable early-stage PC from healthy controls, along with median circulating levels being significantly elevated compared to benign controls and CP. Furthermore, when utilized in combination with CA19-9, diagnostic accuracy was improved significantly for resectable PC cases compared to healthy controls[37]. When combining measurements of CA19-9 assay with detection of CA19-9 on MUC5AC and MUC16, the sensitivity of PC detection improved, with greater sensitivity and near 100% specificity achieved[182].

PAM4: PAM4 antibody is a monoclonal antibody which binds to large-size mucin, and it has been previously been reported that expression of the PAM4-reactive antigen on immunohistology may provide a method for early detection of PC[183-185]. The PAM4 antigen is absent from normal pancreatic tissue or pancreatic tissue associated with benign disease[186]. A 2012 study conducted by Gold *et al*[187] reported the overall sensitivity of PAM4 detection of PC at 75%, with associated high discriminatory power with respect to benign disease, however this has yet to be replicated.

Heat shock protein 27: Heat shock protein 27 (HSP27) is a molecular chaperone which acts to prevent aggregation of misfolded proteins, along with playing a role in the degradation of these proteins[188]. Additionally, it also plays a role in promoting tumour metastasis[189]. In patients diagnosed with PC, HSP27 detection in serum has

been demonstrated to have a sensitivity of 100% and specificity of 84%, however a lack of specificity is highlighted by elevated levels also being reported in CP and cannot be recommended as a diagnostic biomarker in PC[190,191].

CAM17.1: CAM17.1 monoclonal antibody is a monoclonal antibody which detects a mucous glycoprotein that is specific for intestinal mucous, also known as CAM17.1. CAM17.1 is overexpressed in PC but has a low sensitivity and specificity of 78% and 76% respectively in diagnosing PC[192,193].

Fucosylated haptoglobin: Recently fucosylated haptoglobin (Fuc-Hpt) has emerged as a novel biomarker in PC, where it has been demonstrated to be almost equivocal to CA19-9 on ROC curve analysis and also correlates with disease stage[194]. Although this does demonstrate promise as a diagnostic biomarker, it is postulated that Fuc-Hpt is produced by metastatic deposits in the liver, and as such lacks utility in the diagnosis of early stage disease, but rather is able to identify liver metastasis that may not be detected on radiological assessment[195].

Serum amyloid A: Serum amyloid A (SAA) is an acute phase protein which has previously been implicated in a number of disease processes, however with regard to malignancy Yokoi *et al*[196] reported levels of SAA to be elevated in patients with PC compared to controls, although a sensitivity of 96.5% was observed for the detection of PC, and a specificity of 31.9% highlights a shortcoming in its use as a potential diagnostic biomarker.

Aminopeptidase N: Aminopeptidase N (APN/CD13) is a membrane bound metalloproteinase which is expressed in a number of different tumour types and cells, and has been suggested to play a role in tumor progression, proliferation, invasion and angiogenesis[197-199]. APN/CD13 was first evaluated in 2016 by Pang *et al*[200] where an AUC of 0.904 was reported in differentiating PC from benign pancreatic tumours, CP and healthy controls, however this study was limited in its size.

M2-pyruvate kinase: M2-pyruvate kinase (M2-PK) is a glycolytic enzyme that has been demonstrated to have a role in cancer metabolism[201,202]. Initially evaluated in 2004, serum M2-PK was reported to be elevated in patients with PC with a sensitivity and specificity of 85% and 41% respectively, which was subsequently validated in 2008 however elevation was also seen in patients with CP thus highlighting a lack of specificity associated with its implementation as a diagnostic biomarker[203,204].

Apolipoprotein isoforms: Apolipoproteins (APOs), which are produced in the liver and intestine, act as lipid carriers, and in doing so, act as ligands for cell membrane receptors, enzyme cofactors and structural components of lipoproteins (after binding to lipids)[205]. A large number of APOs have been reported to have a role in malignancy with serum APOA2, APOC1, APOC2 and APOE being implicated in PC diagnosis and prognosis.

APOA2, specifically APOA2-ATQ/AT has been demonstrated to be able to distinguish patients with early stage PC compared to healthy controls as well as identifying patients at high risk of pancreatic malignancy. The AUC value for APOA2-ATQ/AT was superior compared to CA19-9 in detecting early stage PC[206]. APOA2 was prospectively evaluated in 2019 where it was identified to be useful when utilized in combination with CA19-9 to improve detection of PC up to 18 mo prior to diagnosis and was suggested to be a useful first measure of PC detection prior to imaging[207]. This was built upon in 2020, where APOA2-ATQ/AT was implemented in a screening cohort in which an elevated level resulted in a PPV of 33.3% for the diagnosis of PC [208].

APOC1 has been implicated in PC where in pre-operative serum, higher levels were reported to correlate with poor prognosis highlighting the potential role of APOC1 as contributing to aggressiveness in PC[209]. Similarly, APOC2 was investigated by Xue *et al*[210] who reported that serum levels independently predicted survival in patients diagnosed with PC.

Serum APOE has been demonstrated to have a sensitivity and specificity of 76.2% and 71.4% respectively for distinguishing patients with PC compared to controls[211, 212]. This study published a superior sensitivity of APOE in diagnosing PC to CA19-9, however it lacked specificity in the diagnosis and was proposed that utilization in combination with CA19-9 could prove beneficial in the future[211]. More recently, when combined in a biomarker panel with inter-alpha-trypsin inhibitor heavy chain H3 (ITIH3), APOA1, APOL1 and CA19-9, a sensitivity and specificity of 95% and 94.1% respectively was reported for the diagnosis of PC[213].

Serum growth factors

Transforming growth factor-beta: According to the findings of Yako *et al*[214] there is a lack of a definitive consensus on the role of transforming growth factor-beta (TGF-β) as a diagnostic biomarker in PC, with serum levels varying in those diagnosed with the malignancy. In addition to this TGF-β has also been implicated in the diagnosis of PC where it has been demonstrated to be elevated in serum samples compared to benign controls, while high levels in serum also significantly correlated with reduced patient survival[215].

Vascular endothelial growth factor: Vascular endothelial growth factor (VEGF) has been reported to have an important role in PC development, while VEGF-A expression has been reported be an important predictor for both distant metastasis and poor prognosis in PC[216]. There is a lack of data affirming the role of serum VEGF as a diagnostic biomarker for PC, with biliary VEGF considered a more accurate diagnostic modality[217].

Fibroblast growth factor 10/keratinocyte growth factor-2: Fibroblast growth factor 10/keratinocyte growth factor-2 (FGF-10/KGF-2) is a regulator of the pancreatic epithelial progenitor cell proliferation and has been implicated in pancreatic morphogenesis along with epithelial mesenchymal transition[218,219]. FGF-10/KGF-2 has been demonstrated to be significantly overexpressed in the sera of patients diagnosed with PC pre-treatment compared to controls, in conjunction with a number of other novel cytokine candidate markers[138].

Platelet-derived growth factor: There is limited data pertaining to the use of platelet-derived growth factor (PDGF) in the diagnosis of PC, however it has been proposed in a panel including IP-10, interleukin (IL)-6 and CA19-9 which demonstrated diagnostic superiority in the discrimination of PC patients from patients with benign disease both in a training and independent test set[220].

Tumour specific growth factor: There is limited data pertaining to the role of tumour specific growth factor (TSGF) in the diagnosis of PC, with a single centre study reporting an increase in specificity for PC when TSGF is used in combination with CA242 and CA19-9 while another study assessed the utility of TSGF as a monitor of response to treatment[221,222].

Serum cytokines and chemokines

Macrophage inhibitory cytokine-1/Growth Differentiation Factor-15: Macrophage inhibitory cytokine-1/Growth Differentiation Factor-15 (MIC-1/GDF15) is a distant member of the TGF-β superfamily of cytokines that has been implicated with inflammation and carcinogenesis, along with serum elevation being detected in a number of pathologies including heart failure and renal failure[223-226].

A meta-analysis published in 2018 aimed to compare MIC-1/GDF15 to CA19-9 as a diagnostic biomarker in PC, identifying fourteen studies with a total of 2826 participants. MIC-1/GDF15 was reported to have a sensitivity of 80% and specificity of 88%, and a diagnostic odds ratio (DOR) of 24.57 which was superior to CA19.9 (DOR = 17.76). In addition to this the AUC of MIC-1/GDF15 in diagnosing PC was 0.8945, which was moderately superior to CA19.9. The conclusion from this study was that MIC-1/GDF15 had comparable diagnostic accuracy to CA19-9, however it was noted that there was marked heterogeneity between studies and that the results should be interpreted with caution[227].

With regard to PC, the authors of this study have recently demonstrated that in a prospective PC screening cohort deemed to be high risk for developing PC based on familial and genetic factors, MIC-1/GDF15 had moderate predictive capacity for patients who subsequently were diagnosed with PC on endoscopic ultrasound (EUS) and biopsy. However, the participants enrolled were considered high risk for developing PC, highlighting a potential issue with generalising the results of this study[228].

ILs: ILs are cytokines that constitute a substantial proportion of those cytokines present in the tumor microenvironment. With regards to their role as diagnostic biomarkers in PC, a considerable number of cytokines have been evaluated in patients diagnosed with PC with variable results (Table 1). There is heterogeneity between studies with insufficient evidence to support their use in routine clinical practice as diagnostic biomarkers, with previous studies demonstrating a lack of diagnostic capacity for PC compared to CRC or benign disease[235].

Oncostatin M (OSM) forms part of the IL-6 cytokine family and has been implicated in promoting epithelial mesenchymal transition, along with being linked to a number of solid organ malignancies[236-238]. Serum levels of OSM have been found to be significantly elevated in patients with PC compared to controls in a single centre study limiting generalisability[138]. There is limited data on the utility of CXC motif ligand 8 (CXCL8)/IL-8 as a diagnostic biomarker in PC. In a relatively small cohort study CXCL8 seems to be superior to CA19-9 and CEA[239].

TNF-α: There is variability in the data pertaining to TNF-α as a diagnostic biomarker in PC. Although the majority of studies report elevated levels of TNF-α in serum compared to healthy controls, a lack of specificity is highlighted as a pitfall in its routine use as a diagnostic biomarker[240-243].

Macrophage colony-stimulating factor: Serum macrophage colony-stimulating factor (M-CSF) has been demonstrated to be elevated in patients with PC compared to controls, along with correlating with advanced stage disease and with non-resectable tumors. Aside from those studies included in the 2016 systematic review published by Yako *et al*[214] there is limited published literature assessing the value of M-CSF as a serological biomarker in the diagnosis of PC.

CXCL11/interferon inducible T cell alpha chemokine: CXCL11 is a CXC chemokine which stimulates the phosphorylation of mitogen-activated protein kinase kinases pathways, resulting in cellular proliferation and prevention of apoptosis[244]. Initially evaluated in 2014, serum CXCL11 was found to be over-expressed in patients with PC compared to controls highlighting a potential role as a diagnostic biomarker, in addition to having a predictive role for gemcitabine and erlotinib treatment response in patients with PC[138].

Stem cell factor: Stem cell factor (SCF) is a ligand that is involved in cell proliferation, differentiation and cell survival, and aside from normal cellular physiology, SCF has been implicated in PC and CRC, with serum levels being noted to be elevated in PC compared to healthy controls, however studies are limited[138,245-248].

Eotaxin: Eotaxin is a protein which is implicated in the recruitment of eosinophils into inflammatory sites which has also been implicated in malignancy[249]. Serum eotaxin was assessed by Zeh *et al*[250] in a single centre study in 2005 in conjunction with hepatocyte growth factor, monocyte chemoattractant protein-1 and CXCL10, were it was able to distinguish PC from healthy controls with a sensitivity of 85.7% and specificity of 92.3%, which was superior to CA19-9.

Serum adhesion molecules

CEA-related cell adhesion molecules: CEA-related cell adhesion molecules (CEACAM) proteins belong to the immunoglobulin supergene family comprised of a variable-like domain as well constant C2-like Ig domains which are required for functionality as well as adhesion. The most well-known CEACAMs related to malignancy are CEACAM1, CEACAM5 (more commonly known as CEA), and CEACAM6. Both CEACAM5 and CEACAM6 are associated with the membrane through a glycosylphosphatidylinositol linkage, while CEACAM1 is anchored to the cellular membrane by transmembrane domains. CEACAM1 have been previously demonstrated to be elevated in a number of tumor entities including PC, however a lack of sensitivity and specificity has been cited as a barrier to its use[217,251-254]. More recently, the role of CEACAMs, including CEACAM1 has shifted from diagnosis to treatment, with CEACAM1 being implicated in cancer immunotherapy[255].

CEACAM6 is a cell surface adhesion receptor that has been previously reported to modulate the ECM in PC[256]. Expression of CEACAM6 was noted in 92% of PC specimens assessed in a 2005 study[257]. Although relatively specific for PC on serum analysis, there is scant evidence to suggest the CEACAM6 as a serological biomarker is useful in the detection of PC with a shift in focus to disruption of CAECAM6 as a therapeutic option in PC[258]. CEACAM5, or CEA, has been demonstrated to have limited efficacy in the diagnosis of PC as described previously, due to it being overexpressed in a number of solid organ malignancies[259,260].

ICAM-1: ICAM-1 is a glycoprotein that functions in cell-cell and cell-ECM adhesion, along with acting as a macrophage chemoattractant[261]. Serum ICAM-1 has been previously evaluated in a number of studies, where it has been demonstrated to be superior to CA19-9 in PC diagnosis. Although preliminary studies have demonstrated promise, its inability to distinguish between early and late-stage PC have been

identified as a potential dilemma limiting its implementation as a screening and diagnostic biomarker[262,263].

Serum non-coding RNAs

Long non-coding RNAs: Long non-coding RNAs (LncRNAs) belong to a group of RNAs that are longer than 200 nucleotides and are not translated into proteins. These RNAs are abundant in cells, and were previously thought to be of minimal value with minimal influence on biological behaviour[264]. This belief has however changed over the past 10 years, with more recent data suggesting that lncRNAs have a diverse range of function, including chromatin modification, gene transcription, post-translational modification and regulation of intracellular signalling pathways[265]. In addition to this, they play a role in either the promotion or suppression of tumor growth, through involvement in intracellular signalling pathways[266] (Table 2).

LncRNA in PC have the potential to modulate both intrinsic and acquired chemo-resistance. Additionally, lncRNA also possess the capacity to act as a miRNA sponge, to perform chromatin remodelling, and promote gene transcription in candidate tumour suppressor genes by binding to gene promotors[267-270]. In terms of the role of lncRNAs as a diagnostic marker in PC a number of candidates have been evaluated with mixed results, and studies are limited to single cohort studies yet to be validated [271]. Perhaps the most promising study to date in search for a lncRNA biomarker was published in 2020, which utilized analysis of the extracellular vesicle lncRNA profile by extracellular vesicle lncRNA sequencing in patients diagnosed with PC and CP. This was performed utilizing a support vector machine algorithm to detect a d-signature for eight different extracellular vesicular long RNA. This study demonstrated that through utilisation of the d-signature, an AUC of 0.949 was able to be achieved in identifying resectable stage I/II PC, while also demonstrating superiority when compared to CA19-9 when distinguishing PC from CP[272].

MiRNAs: MiRNAs are noncoding 20-25 nucleotide endogenous RNA sequences who regulate gene expression and are able to regulate the biological function of many tumors[273]. MiRNAs have become prominent in the field of oncology in the diagnosis, prognosis and monitoring of therapy of cancer. In addition to their presence in serum, miRNAs have also been detected in cerebrospinal fluid, breast milk, saliva and urine[274,275]. Although the method through which miRNA are released into the peripheral circulation from active malignancies is still being determined, their ability to withstand severe conditions along with extended storage highlights an exciting potential diagnostic biomarker. Due to the lack of a gold-standard diagnostic biomarker for PC, research into the efficacy of miRNA as a diagnostic biomarker in PC has progressed rapidly in the past decade with a large number of candidate miRNA biomarkers utilized in serum for the detection of PC as demonstrated in Table 2. Perhaps the most comprehensive analysis to date reviewing candidate miRNAs utilized in PC comes from a large meta-analysis published in 2018 encompassing 80 studies which detected miRNA in blood (including whole blood, serum and plasma samples that concluded that candidate miRNA biomarkers are useful in PC, particularly when used in combination, however no standing panel was reported to exist at this stage[276].

The rapid expansion of miRNA utilization in serum in the diagnosis of PC highlights its potential value as a future diagnostic biomarker modality which could be implemented into routine clinical practice, however determination of which miRNA possesses the greatest diagnostic accuracy is required. Panel based assays represent a very attractive methodology for miRNA detection which have been identified as having superior diagnostic accuracy, however further validation of specific candidate miRNAs is required.

Serum liquid biopsy

Exosomes: Exosomes are membrane-bound nano-capsules that transfer molecules between cells[308]. Their role in the diagnosis of PC is limited to only a handful of studies which were recently included in a relatively small systematic review meta-analysis which also assessed circulating tumor cells (CTCs) and cell-free DNA (cfDNA). In six papers included, exosomes were found to have strong diagnostic value with an AUC of 0.9819[309]. It was postulated that they possessed value in the field of PC detection due to pancreatic cells possessing a strong exocrine function, along with the high activity of PC cells. A number of different types of exosomes were analyzed as demonstrated in Table 3.

Table 2 Serum based non-coding RNA biomarkers implicated in the diagnosis of pancreatic cancer

Type	Candidate marker
LncRNA	LINC-PINT[277], SNHG15[278,279], LINC01238[280], ABHD11-AS1[281], HULC[282,283], UFC1[284]
MiRNA	miR-21[285-289], miR-25[288,290,297], miR-210-3p[289], miR-29a[290], miR-19a[290], miR-210[285,291], miR-155[285,292], miR-499a-5p[293], miR-125a-3p[294], miR-6893-5p[294], miR-125b-1-3p[294], miR-6075[294], miR-6836-3p[294], miR-1469[294], miR-6729-5p[294], miR-575[294], miR-204-3p[294], miR-6820-5p[294], miR-4294[294], miR-4476[294], miR-4792[294], miR-196a[285,295], miR-18a[296,297], miR-10b[292-298], miR-106b[292], miR-642-3p[299], miR-885-5p[299], miR-22-3p[299], miR-34a[286], miR-191[297], miR-451a[300], miR-121-5p[298], miR-30c[298], miR-483-5p[290,297], miR-1290[301,302], miR-24[290,297,301], miR-134[301], miR-146a[301], miR-378[301], miR-484[301], miR-628-4p[301], miR-1825[301], miR-1246[302], miR-482-3p[287], miR-16[295], miR-27a-3p[303], miR-192[304], miR-885-5p[299], miR-22-3p[299], miR-642b-3p[299], miR-492[305], miR-663a[305], miR-194[304], miR-223[306], miR-774-5p[307], miR-409-3p[307], miR-128-3p[307], miR-20a[290,297], miR-27a[297], miR-29c[297], miR-30a.5p[297], miR-323.3p[297], miR-345[297]

MiRNA: MicroRNA; LINC-PINT: Long intergenic non-protein coding RNA, P53 induced transcript; SNHG15: Small nucleolar RNA host gene 15; ABHD11-AS1: ABHD11 antisense RNA 1; HULC: Highly up-regulated in liver cancer.

Table 3 Serum based 'liquid biopsy' biomarkers implicated in the diagnosis of pancreatic cancer

	Biomarkers
Exosomes	Exosomes: GPC1[310,313], miR-10b[310], miR-30c[310], miR-181-a[310], miR-let7a[310], miR-17-5p[311], miR-21[311], miR-1246[312], miR-4644[312], miR-3976[312], miR-4306[312]
ctDNA	*KRAS*[314-317], *ADAMTS1*[318], *BNC1*[318]
CTC	CAPI+/CD45-[319], CK+[319], CEA+[319], CD45-/DAPI+/CEP8[320], CD45[321], CCK19[321], Pdx-1[321], Kras mutation[322], CEP8[323], CK[323], CD45[323], DAPI[323], chromosome 8[324], Folate-receptor positive CTCs[326]

Tspan8: Tetraspanin 8; EpCAM: Epithelial cell adhesion molecule; MET: mesenchymal-epithelial transition factor; CD104: Integrin 4-beta; GPC1: Glypican 1; GNAS: Guanine Nucleotide binding protein; KRAS: KRAS Proto-Oncogene, GTPase; ADAMTS1: A disintegrin and metalloproteinase with thrombospondin motifs 1; BNC1: Basonuclin 1; CD45: Leukocyte common antigen; CK19: Cytokeratin 19; Pdx-1: Pancreatic and duodenal homeobox 1; ADAMTS1: A disintegrin and metalloproteinase with thrombospondin type 1 motif, member 13; BNC1: Zinc finger protein basonuclin-1.

CTCs: Initially identified in 1896 in metastatic breast cancer, CTCs are cells that are shed from primary tumor or metastatic deposits which enter the bloodstream directly and can be detected forming what is known as a real-time "liquid biopsy"[325]. In a recently published systematic review and meta-analysis, seven articles were identified which utilized CTCs in the diagnosis of PC, of which multiple methods of detection were used highlighting heterogeneity between study methodology. The pooled sensitivity and specificity of CTCs were 74% and 83% respectively, with and AUC of 0.8166. The authors' conclusion was that CTCs had moderate diagnostic value in PC [309].

CTCs demonstrated inferiority when compared to exosomes in the systematic review due to their inferior sensitivity and specificity, however their AUC was still deemed acceptable from a diagnostic capacity for PC. Folate receptor positive CTCs have also been implicated as a novel diagnostic biomarker in those patients diagnosed with periampullary malignancy on ligand-targeted polymerase chain reaction demonstrating a significant elevation compared to those with benign pancreatic disease[326]. In addition to this, when utilized in combination with CA19-9, it was reported to have a superior sensitivity and specificity of 97.8% and 83.3% respectively, compared to when used in isolation. CTCs have yet to be utilized in a prospective screening population. Decreased blood flow to malignant pancreatic tissue along with increased CTC accumulation in the liver due to the portal circulation are posed as challenges in the detection of PC related CTCs[327].

Circulating tumor DNA: cfDNA, initially identified in 1948, is fragmented DNA identified in the circulation. It has been applied to many areas of medicine, ranging from prenatal assessment, renal failure, and stroke where it has had mixed results[328-330]. In the case of medical oncology, the detection and utilisation of cfDNA secreted from tumours, referred to as circulating tumor DNA (ctDNA) has been met with a number of challenges, namely the ability to discriminate ctDNA from normal cfDNA, and low levels of ctDNA hampering detection[331].

The diagnostic value of ctDNA in PC has been deemed to be promising with a recent meta-analysis being able to identify seven articles assessing ctDNA in the

diagnosis of PC showed a pooled sensitivity and specificity were 64% and 92% respectively, with an AUC of 0.9478[309]. In this review, ctDNA was deemed inferior to CTCs from a sensitivity perspective, however the AUC was superior in diagnosing PC. This was attributable to the inability to detect low levels of circulating ctDNA in early stages of cancer when overall tumor burden was low, highlighting a dilemma in utilizing this form of diagnostic biomarker in early stages of disease and as a screening modality. A summary of the included ctDNA biomarkers can be viewed in Table 3.

Plasma ctDNA quantification of hot-spot mutations in KRAS and GNAS has also been reported to be useful in predicting tumor burden in patients diagnosed with PC. In addition to this, digital PCR (dPCR) provided accurate tumor-derived mutant KRAS detection in plasma in resectable PC and improved post-resection recurrence prediction compared to CA19-9[332].

URINARY BIOMARKERS

Urine protein biomarkers

Urine proteins have also been established as a means through which PC can be detected, with previous proof-of-concept studies demonstrating that protein signatures associated with PC can be detected in the urine[333]. Radon *et al*[334] were able to build upon this, where they reported that three proteins, lymphatic vessel endothelial hyaluronan receptor 1, REG1A and thyroid transcription factor 1, when combined in a biomarker panel, were able to detect patients with PC with an AUC of 0.89 and 0.92 in training and validation datasets respectively, compared to healthy controls. Although further validation is required, this presents an inexpensive and non-invasive option for screening in patients for PC, and was suggested to be added to the current screening modalities utilized in high-risk patients to determine its efficacy prospectively[334]. Aside from this there is relatively little published with regard to the urinary proteome in the detection of PC and other proteins implicated are limited to single centre cohort studies (Table 4).

Urine non-coding RNA

MiRNA: Urinary miRNA has previously been utilized in the detection of bladder cancer, however, there is scant literature to support the use of urinary miRNA in the detection of PC[339]. In a small British study, Debernardi *et al*[340] were able to demonstrate that miR-143, miR-223 and miR-30e were significantly over-expressed in patients with stage I PC compared to age-matched healthy individuals. MiR-1246 has also been assessed as a urinary biomarker, where significantly higher levels of expression were noted in patients with PC compared to controls, with an AUC of 0.90 which was superior to serum miR-1246 (AUC = 0.87)[18]. Considering the non-invasive capacity of urine sampling, coupled with the rapid expansion and interest in use of miRNA in the detection of malignancy, further studies should aim to determine whether experimental studies can translate into larger prospective clinical studies.

Urine liquid biopsy

Urinary cfDNA: Considering the rapid expansion of the concept of a 'liquid biopsy', the hypothesis that tumour DNA could be detected through the urine with urinary cfDNA originating from the shedding of cells directly from the genitourinary tract or *via* the circulation passing through the kidney and filtering through the glomerulus also known as transrenal DNA has emerged as a method of biomarker detection. Terasawa *et al*[341] were able to detect urine KRAS mutations in 48% of participants diagnosed with PC, which was equivocal with the serum detection rate. This method of detection however is influenced by the patient's underlying kidney function.

Exosomes: More recently, the ratio of miR-3940-5p/miR-8069 in urine exosomes has been implicated in PC. This ratio was noted to be elevated in patients diagnosed with early stage PC, with a sensitivity of 93.0% and PPV of 78.4%[342].

Other urinary markers

Detection of volatile organic compounds (VOCs) is a relatively novel area in malignancy diagnosis, which utilized odors that emanate from urine, breath and faeces. These compounds are produced by bacterial dysbiosis which is secondary to malignancy. Recently Nissinen *et al*[343] were able to demonstrate through using field asymmetric waveform ion mobility spectrometry that patients diagnosed with PC could be distinguished from healthy controls with a sensitivity and specificity of 79%

Table 4 Urinary biomarkers implicated in the detection of pancreatic cancer

Type	Candidate marker
Protein	LYVE1[334], REG1A[334], TTF1[334], TIMP1[335], MMP-2[335], NGAL[336], PGE2 metabolites[337], CD59 glycoprotein (CD59)[338], ANXA2[338], 21 kDA gelsolin fragment[338], S100A9[338]
Liquid biopsy	UcfDNA: KRAS mutation[341]; Exosomal miRNA: miR-3940-5p[342], miR-8069[342]
RNA	MiRNA: miR-143[340], miR-223[340], miR30e[340], miR-1246[18]
Metallomics	Calcium[344], magnesium[344]
Other	VOCs[343]

MiRNA: MicroRNA; ANXA2: Annexin A2; S200A9: Protein S100-A9.

and 79% respectively through the detection of VOCs in the urine. Additionally, the analysis of the metallomic signature of urine is also a relatively uncharted area in the field of PC, with a study published by Schilling *et al*[344] recently demonstrating that in those diagnosed with PC, urine calcium and magnesium were significantly lower compared to healthy controls. They were able to demonstrate through combined analysis that these metals were accurate indicators for metal dyshomeostasis in PC with a sensitivity of 99.5%.

PANCREATIC JUICE BIOMARKERS

Pancreatic juice is usually obtained during the ERCP which is an invasive procedure with potential morbidity and mortality and is not used routinely as a screening procedure. Alternatively, pancreatic juice can be collected during the endoscopy from the duodenum after secretin administration which has the risk of secretin induced pancreatitis and contamination of the sample with duodenal and gastric juice. While attractive, pancreatic juice biomarkers are unlikely to be used in large populational studies but it might be useful in selected cases in which endoscopy or ERCP is indicated (Table 5).

Protein based biomarkers

Protein biomarkers are the most well explored candidate biomarkers in the medium of pancreatic juice. Conventional markers utilized in serum, such as CA19-9 and CEA, have been implicated in pancreatic juice where the sensitivity of CA19-9 is questionable, while CEA demonstrated merit in predicting malignant transformation of IPMNs along with the diagnosis of PC[345-352]. Aside from these biomarkers, a large number of proteins have been assessed in the pancreatic juice of patients with variable results, however considering that evidence supporting these biomarkers is limited to only a handful of small cohort studies, their implementation as a diagnostic tool is not recommended.

Although mucins have been extensively investigated in the diagnosis of PC, with regard to pancreatic juice there is limited literature published on its value. Levels have been demonstrated to be elevated in the case of MUC1, and KL-6 mucin, a type of MUC1, was investigated by Matsumoto *et al*[354] and reported to be significantly elevated in the pancreatic juice of patients with PC and IPMC compared to inflammatory lesions and IPMNs however its specificity was less than desirable.

Non-coding RNA

When compared to serum and saliva, pancreatic juice has proved to be less fruitful with regard to candidate miRNA biomarkers in PC diagnosis. Both miR-21 and miR-155 have been demonstrated to be elevated in the pancreatic juice of patients diagnosed with PC compared to CP[362], while Wang *et al*[363] was also able to report a specificity of 88% and sensitivity of 87% when four circulating miRNAs in pancreatic juice (miR-205, miR-210, miR-492 and miR-1427) were used in combination for detecting PC. In addition to miRNA assessed in pancreatic juice, MSLN mRNA has also been implicated in the diagnosis of PC on pancreatic juice[364].

Table 5 Pancreatic Juice biomarkers implicated in the detection of pancreatic cancer

Type	Candidate marker
Protein	CA19-9[345-347,349], MIC-1[349], NGAL[349], CEA[347,348,350-352], AMYP[353], PRSS1[353], glycoprotein GP2-1[353], CCDC132[353], REG1A[353], REG1B[353], REG3A[353], LIPRP2[353], KL-6/MUC1[354], CPA5[355], inactive LIPRP1[355], KLK1[355], HBD[355], TTR [355], S100P[356], MMP-9[357], MMP-7[155], DJ-1[357] A1BG[357], PAP-1[358], AGR2[359], IL-8[360], Cathepsin E[361]
RNA	MiRNA: miR-21[362], miR-155[362] , miR-205[363], miR-210[363], miR-492[363], miR-1427[363]; mRNA: mesothelin[364]; Other: hTERT [365,366], telomerase activity[367-369]
Liquid biopsy	Exosomes: CEACAM1[371], CEACAM 5[371], tenascin C[371], MMP7[371], LAMB3[371], LAMC2[371], MUC1[372], MUC4[372], MUC5AC[372], MUC6[372], MUC16[372], CFTR[372], MDR1[372], ex-miR-21[373], ex-miR-155[373]; Methylated DNA: KRAS[374,377], ppENK[375,376], p16[375,376], Cyclin D2[376], FOXE1[376], NPTX2[376], TFPI2[376], CD1D[377], KCNK12[377], CLEC11A[377], NDRG4 [377], IKZF1[377], PKRCB[377], MUC1[378], MUC2[378], MUC4[378]

MiRNA: MicroRNA; PRSS1: Trypsin-1; CPA5: Carboxypeptidase A5; KLK1: Kallikrein-1; HBD: Hemoglobin Subunit Delta; LAMB3: Laminin subunit beta-3; CFTR: Cystic fibrosis transmembrane conductance regulator; MDR1: Multidrug resistance protein 1; KCNK12: Potassium channel, subfamily K, member 12; CLEC11A: C-Type lectin domain containing 11A; NDRG4: NDRG family member 4; IKZF1: Ikaros family zinc finger protein 1 gene; PKRCB: Protein kinase C beta; FOXE1: Forkhead Box E1; NPTX2: Neuronal pentraxin-2.

Liquid biopsy

Telomerase activity and human telomerase reverse transcriptase: Telomerase activity has previously been deemed a promising marker as it was shown to be elevated in pancreatic juice samples of patients with PC[365-367]. Further to this, a recent meta-analysis assessing the diagnostic utility of the four major altered genes in PC (KRAS/CDKN2A/p16, TP53, and SMAD4/DPC4), telomerase activity, and a combination assay, revealed that the most reliable biomarker in diagnosing PC in pancreatic juice samples was telomerase activity[367]. Human telomerase reverse transcriptase (hTERT) is a catalytic subunit of telomerase, and the detection of mRNA for hTERT has been postulated to aid in the diagnosis of malignancies including PC. hTERT was first detected in 10 of 11 patients diagnosed with invasive PC on pancreatic juice sampling[368]. This was further validated by Nakashima *et al*[369] and was additionally assessed in a recent systematic review assessing the role of hTERT which reported that telomerase reactivation played a significant role in the development of hepatobiliary and pancreatic tumors, along with being a diagnostic biomarker for PC[369,370].

Methylated DNA: Mutations in the KRAS oncogene are present in over 90% of resected PC specimens, with the vast majority of these mutations occurring in KRAS codon 12. A recent meta-analysis published by Patel *et al*[374], encompassing 22 studies aimed to assess the diagnostic accuracy of mutant KRAS detection from pancreatic secretions (mucus, secretions and juice) for the diagnosis of PC. They reported a wide variation in sensitivity (38%-89%) and specificity (13%-100%) for the diagnosis of PC through KRAS mutation testing in pancreatic secretions, with significant heterogeneity in diagnostic accuracy across the included studies. They also assessed whether KRAS mutation detection would be beneficial in diagnosing PC in a screening population, which similarly returned a sensitivity ranging from 21%-86%, however specificity improved remarkably to 82%-100%[374]. In addition to KRAS, Methylated ppENK and p16 were reported to be present in pancreatic juice in 90.9% and 18.2% respectively of patients diagnosed with PC, and due to normal pancreatic juice not containing methylated forms of this DNA, their presence was postulated to suggest the presence of PC[375]. Other markers investigated in single centre studies are shown in Table 5. MUC1 was also assessed in conjunction with MUC2 and MUC4 in 2014. Yokoyama *et al*[378] reported that DNA methylation status of MUC1, MUC2 and MUC4 was useful for the differential diagnosis of human pancreatic neoplasms, with a sensitivity and specificity of 87% and 80% for PC.

PANCREATIC CYST FLUID BIOMARKERS

Pancreatic cysts (PCy) are proving to be a promising area in the field of specimen sampling for biomarker identification. PCy incidence increases with age, with the most common cyst types including IPMN, mucinous cystic neoplasms (MCN), serous cystic neoplasms, and pseudocysts[379-381] (Table 6).

Table 6 Pancreatic cyst fluid biomarker studied in relation to high grade dysplasia and pancreatic cancer diagnosis

Type	Candidate marker
Protein	CEA[383,384,399,402-407], Glucose[385], MUC4[386,412], PGE2[387,388], IL-1B[386,387], PGE synthetase 2[386], IL-4[389], CA72-4[389], sFASL [389], MMP9[389] AREG[390,391], SPINK1[392], mAB Das-1[393,394], IL-10[395], GM-CSF[395], MUC1[413], MUC2[413], MUC5AC[413]
RNA	MiRNA: miR-21[396], miR-221[396], miR-18a[397,398], miR-24[397,398],miR-30a-3p[397,398], miR-92a[397,398], miR-99b[397,398], miR-106b[397, 398], miR-142-3p[397,398], miR-342-3p[397,398], and miR-532-3p[397,398]
Other	DNA based-*KRAS* mutations[399-407,409-411]*GNAS* mutations[409-411]

MiRNA: MicroRNA; CA72-4: Cancer antigen 72-4; sFASL: Soluble Fas; AREG: amphiregulin; SPINK1: serine peptidase inhibitor kazal type 1; GM-CSF: granulocyte macrophage colony-stimulating factor.

Due to IPMNs and MCNs possessing a risk of developing into PC identification of cyst fluid biomarkers in these pre-malignant lesions help to select which patients to proceed to surgery[382]. The cyst fluid is aspirated during EUS (EUS-FNA) under antibiotic cover and the amount of fluid retrieved depends on the size of the cyst therefore highlighting a potential for insufficient sampling during aspiration. Pancreatic cyst fluid analysis was initially focused on proteins isolated for biomarker assessment, however more recently there has been a transition towards the analysis of non-coding RNA, or miRNA in pancreatic cyst fluid to determine their diagnostic capacity for PC[408].

Proteins analyzed on cyst fluid, for the most part, have been reported to lack specificity in the diagnosis of PC, however mucin analysis, CEA level and VEGF-A on cystic fluid has proved to have efficacy in discriminating premalignant and malignant lesions from benign lesions. MUC4 expression has been implicated in PCy, being elevated in MCN, and has been postulated to assist in early detection of PC[412]. In addition to this, MUC1, MUC2 and MUC5AC have been demonstrated to be upregulated in patients with PC on cytology obtained during EUS-FNA but MUC7 is upregulated in PC and also in IPMN and CP, limiting its specificity in the diagnosis of PC[413,414].

Additionally, DNA-based biomarkers, including KRAS and GNAS, have been evaluated in the context of PC diagnosis and IPMN and noted to be elevated in mucin producing cysts. Recently, supervised machine learning techniques were used to develop a test to guide management of PCy based on clinical features, imaging and cyst fluid genetic and biochemical markers (CompCyst)[415]. Due to invasive nature of cyst fluid collection, the authors recommend that future studies should focus on biomarkers and algorithms that can help select which cysts have malignant potential and should proceed to surgery.

SALIVARY BIOMARKERS

Saliva is an emerging interest in the field of biomarker detection as it provides a non-invasive means through which potential diagnostic biomarkers can be sampled. It has previously been validated in the areas of drug abuse, human immunodeficiency virus infection and hormone assessment, along with detection of oral, breast, lung, ovarian and oesophageal cancer, and has been recently named the "diagnostic window to the body"[416-418] (Table 7).

The analysis of salivary fluid as a means for identification and evaluation of diagnostic biomarkers for PC is in its infancy, with proteomic biomarkers scant in the literature and due to the large amounts of salivary amylase, albumin and immunoglobulins present in saliva, their subsequent sensitivity is hampered in PC diagnosis [419,420]. Given this lack of sensitivity, there has been a shift in focus to RNA based biomarkers, namely LncRNA and miRNA . A recent systematic review reported that PC is the most investigated disease in relation to the utilization of salivary miRNA analysis. This is highlighted by 18 miRNA candidates which have been detected and studied in relation to PC, irrespective of stage, through the medium of saliva. Although miRNA analysis in saliva is in its infancy with regard to PC, the reported specificity in the diagnosis of PC is impressive and warrants further validation. Despite this reported specificity, the aforementioned systematic review concluded that there is marked heterogeneity between studies and as such meta-analysis is unachievable, highlighting the need for further research in this area[421-425].

Table 7 Salivary fluid biomarkers studied in relation to pancreatic cancer diagnosis

Type	Candidate marker
RNA	LncRNA: *HOTAIR*[428], *PVT1*[428]; MiRNA: miR-21[286,423,431], miR-23a[423], miR-23b[423], miR-29c[423], miR-1246[422], miR-4644[422], miR-34a[286], miR-155[286], miR-200b[286], miR-376a[286], miR-216[423], miR-940[424], miR-3679-5p[424], miR-17[425], miR-181b[425], miR-196a[425]
Other	Salivary polyamines: Alanine[427], N_1-acetylspermidine[427], 2-oxobutyrate[427], 2-hydroxybutyrate[427]

MiRNA: MicroRNA.

Aside from proteomic and RNA analysis of saliva, polyamine analysis has also emerged as a potential diagnostic biomarker candidate. Abnormalities in tumor-suppressor genes, deemed to play a key role in PC development, accelerate polyamine synthesis and as such, increased levels have been postulated to be a potential biomarker in PC[426]. Only a single study has assessed polyamines in PC detection with modest diagnostic accuracy[427].

BILIARY FLUID BIOMARKERS

Biliary fluid is a potential source for biomarkers, however due to sampling requiring an invasive procedure, ERCP, there are inherent risks with this mode of acquisition and is not routinely used. Currently the literature is limited to protein-based biomarkers, non-coding RNA markers and methylated DNA as a method of liquid biopsy with a recent meta-analysis highlighting minimal literature on biliary miRNA markers utilized in PC diagnosis[445] (Table 8).

There have been mixed results from these studies with a lack of large prospective studies to determine the validity of these biomarkers in clinical use. Although some biomarkers display merit in the early phases of clinical research, their role has also been deemed to be of value in the diagnosis of indeterminate biliary strictures thus highlighting a potential lack of sensitivity in the diagnosis of PC. Given the invasive nature of acquisition, less intrusive methods of biomarker acquisition should be considered for future research.

FAECAL BIOMARKERS

The concept of being able to detect PC biomarkers in stool is due to the large amount of pancreatic juice produced and excreted into the bowel on a daily basis, highlighting the potential that that precancerous or molecular changes indicative of a malignant process can be detected in faeces[447] (Table 9).

Faecal protein biomarkers

Adnab-9: Adnab-9 is a murine monoclonal antibody that has previously been implicated in the diagnosis of gastrointestinal tumors[448,449]. Adnab-9 detection in stools has a sensitivity and specificity of 80% and 87% for detection PC[450,451].

Faecal non-coding RNA

MiRNA: Faecal miRNA detection as a diagnostic biomarker has been utilized in CRC where although the environment has deemed to be more hostile than blood, miRNAs have been demonstrated to remain intact and stable for detection due to being packaged in exosomes. Faecal miRNA detection only requires 1 g of faeces in a sample, therefore presents itself as an efficacious modality as a screening test. Although there is only scant literature describing faecal miRNA analysis as a biomarker in PC[19,452,453], certain candidate markers demonstrate promise however there is heterogeneity between studies. Further studies are required to determine the relationship of faecal miRNA expression in PC to determine whether a candidate marker can be utilized in a screening population.

Faecal liquid biopsy

Faecal mutant *KRAS*: Initially detected in 1994 by Caldas *et al*[454], the presence of *K-ras* mutation in stool in patients with PC has proved to be an area of promise with regard to a non-invasive method of detection, and has also been explored in

Table 8 Biliary fluid diagnostic biomarkers studied with relation to pancreatic cancer

Type	Candidate marker
Protein	VEGF[217,429], CA19-9[431], CA125[432], CA72-4[432], CEA[432,433], sLR11[434], MUC4[435], IGF-1[217,430], NGAL[436-439], CEAM6[436,440], LG3BP[436], MMP7[436], MUC5B[436], MCM5[441,442], Trypsinogen-1[443], Trypsinogen-2[443]
Liquid biopsy	Methylated DNA: *TFP12*[444], *NPTX2*[444], *CCND2*[444]
RNA	MiRNA: miR-10b[292,445], miR-106b[292,445], miR-30c[292,445], miR-155[292,445], miR-212[292,445], miR-1247[446], miR-200a[446], miR-200b[446]

MCM5: Minichromosome maintenance protein 5; NGAL: Neutrophil gelatinase-associated lipocalin; sLR11: Soluble LDL receptor relative with 11 ligand-binding repeats; IGF1: Insulin-like growth factor 1; LG3BP: Galectin-3-binding protein; CEAM6: Carcinoembryonic cell adhesion molecule 6; TFP12: Methylated tissue factor pathway inhibitor 2; NPTX2: Neuronal pentraxin II gene; CCND2: G1/S-specific cyclin-D2; VEGF: Vascular endothelial growth factor; CEA: Carcinoembryonic antigen; MiRNA: MicroRNA.

Table 9 Faecal diagnostic biomarkers implicated in pancreatic cancer

Type	Candidate marker
Protein	Adnab-9[450,451]
RNA	MiRNA: miR-181b[452], miR-210[452], miR-155[453], miR-216a[453], miR-196a[452,453], miR-143[453]
Liquid biopsy	Mutant *KRAS*[454,455], mBMP3[456]

MiRNA: MicroRNA.

combination with methylated bone morphogenetic protein 3 (mBMP3)[454-456].

mBMP3: There is scarce literature regarding the role of BMP3 in the diagnosis of PC with a single study in 2011. Stool mBMP3 use as a biomarker for PC was first assessed in 2012, where it was able to detect 51% of PCs, compared to mutant *KRAS* which detected 50%. The AUC for mBMP3 was 0.73, however when used in combination with mutant *KRAS*, an AUC of 0.85 was achieved highlighting a potential option for non-invasive biomarker testing in a prospective cohort[456].

CONCLUSION

The literature is diverse with regard to biomarkers in the diagnosis of PC, with variation both in the medium utilized (serum, urine, saliva, pancreatic juice, cyst fluid analysis, faeces), along with the type of biomarker detected (miRNA, exosomes, proteins, CTCs, ctDNA) as demonstrated through this review, encompassing over 300 different diagnostic biomarkers in a variety of mediums. The current diagnostic biomarker utilized in the routine diagnostic work-up of PC is CA19-9, however this lacks sensitivity highlighted by phenotypic variation in the Lewis blood group antigen. Current research has focused on miRNA, ctDNA and CTCs in the detection and subsequent diagnosis of PC in experimental or feasibility studies with mixed results so far. Perhaps the most promising area of diagnostic biomarker discovery in the field of PC is the utilisation of diagnostic panels comprising a number of candidate markers rather than a single candidate protein or miRNA. These panels have proved to be efficacious in their diagnostic capacity for PC and as such should be further explored in prospective multi-centre studies to prove generalizability of results across different population groups. Very minimal research has been conducted evaluating biomarkers as a screening tool, with the low incidence of PC in the general population being cited as a barrier. This should be further explored to determine whether these candidate markers can be used as part of a screening program. A small number of studies have assessed the role of biomarkers in high-risk populations part of PC screening programs, however further research is required to determine whether their results can be extended to the general population. Future studies should aim to capitalize on the non-invasive nature of salivary, urinary, faecal and serum testing, as ultimately at a population level these are the most implementable modalities of testing and use cyst analysis and pancreatic juice in undetermined pancreatic lesions when

surgery is contemplated. Although we are yet to find the elusive 'golden ticket' for diagnosing PC, translational research is constantly opening up new doors in the search for a diagnostic biomarker that will help select the patients who need further investigations aimed at detecting PC early, similar to a positive FOBT prompting further assessment with a colonoscopy.

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REVIEW

Non-occlusive mesenteric ischemia: Diagnostic challenges and perspectives in the era of artificial intelligence

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Abstract

Acute mesenteric ischemia (AMI) is a severe condition associated with poor prognosis, ultimately leading to death due to multiorgan failure. Several mechanisms may lead to AMI, and non-occlusive mesenteric ischemia (NOMI) represents a particular form of AMI. NOMI is prevalent in intensive care units in critically ill patients. In NOMI management, promptness and accuracy of diagnosis are paramount to achieve decisive treatment, but the last decades have been marked by failure to improve NOMI prognosis, due to lack of tools to detect this condition. While real-life diagnostic management relies on a combination of physical examination, several biomarkers, imaging, and endoscopy to detect the possibility of several grades of NOMI, research studies only focus on a few elements at a time. In the era of artificial intelligence (AI), which can aggregate thousands of variables in complex longitudinal models, the prospect of achieving accurate diagnosis through machine-learning-based algorithms may be sought. In the following work, we bring you a state-of-the-art literature review regarding NOMI, its presentation, its mechanics, and the pitfalls of routine work-up diagnostic exams including biomarkers, imaging, and endoscopy, we raise the perspectives of new biomarker exams, and finally we discuss what AI may add to the field, after summarizing what this technique encompasses.

Key Words: Mesenteric ischemia; Biomarkers; Critically ill; Machine learning; Artificial intelligence

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Core Tip: In this review we focus on non-occlusive mesenteric ischemia and discuss the challenges of a reliable diagnosis, which requires several simultaneous elements, including physical examination, biomarkers, and imaging elements. While taken individually these elements do not provide sufficient diagnostic accuracy, a multimodal approach relying on artificial intelligent algorithms may increase speed and accuracy in recognizing this rare but severe condition.

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INTRODUCTION

Acute mesenteric ischemia (AMI), due to inadequacy between oxygen demand and supply in the digestive tract, is a life-threatening emergency[1]. This term encompasses several entities that differ regarding their initial trigger of gut ischemia but ultimately converge towards digestive and systemic complications such as tissue necrosis, perforation, bacterial translocation, and eventually, death due to multiorgan failure. Contrary to most conditions, AMI is associated with a poor prognosis, which has not improved in the last decades. Mortality ranges around 80% and mostly depends on early diagnosis and adequate treatment.

Diagnosis of AMI secondary to large vessels occlusion mainly relies on imaging, including contrast-enhanced abdominal computed tomography (CT) scan, allowing the identification of the occluded vessel (or vessels) in order to choose between different revascularization options (interventional, surgical, or medical treatment).

While obstructive AMI has been reported at length, and their management is supported by evidence-based guidelines[2], AMI occurring in the absence of major vascular occlusion, non-occlusive mesenteric ischemia (NOMI), frequently raises diagnostic and therapeutic challenges. Indeed, NOMI often occurs as the consequence of a critical condition[3]. The diagnosis is often suspected in the intensive care unit (ICU) in the context of a patient's clinical condition worsening after a prior episode of profound and acute circulatory failure, such as a successfully resuscitated cardiac arrest, cardiopulmonary bypass surgery, as well as septic, hypovolemic, or cardiogenic shock. Reported mortality rates are extremely high, and time to diagnosis represents a key factor for improving its associated prognosis[4,5].

Several leads have been pursued to achieve this goal, including the development of new biomarkers as well as new multimodal tools. In the last decade, the advent of artificial intelligence (AI) allowed the facilitation of complex diagnoses relying on imaging.

In the following work, we bring you a state-of-the-art literature review regarding NOMI, its presentation, its mechanics, the pitfalls of routine work-up diagnostic exams, and perspectives in new biomarker exams and finally discuss what AI may add to the field. For brevity, we did not cover therapeutic management.

EPIDEMIOLOGY, MECHANISMS, AND MANAGEMENT OF NOMI

In contrast to AMI secondary to large vessels occlusion, NOMI was initially poorly understood. Nearly 80 years ago, first reports of NOMI described intestinal gangrene secondary to low cardiac output but without evidence of either arterial or venous occlusion[6,7]. As of today, only case-series and retrospective cohort studies report these severe events and in a selected population. One exception reports epidemiological data in a general population[8]. This Swedish population-based study was performed between 1970 and 1982 and suggested a population-based incidence of fatal NOMI of 2/100000 person-years. From 23446 systematic autopsies, 62 fatal NOMI cases were identified. After clinical data records were investigated, these patients were more likely to have suffered from fatal cardiac failure, atrial fibrillation, and recent

surgery. Of note, necroptic examination often showed concomitant infarction of other visceral organs such as liver, spleen, and kidneys suggesting a state of global organ hypoperfusion. Through non-recent retrospective monocentric surgical case-series, NOMI ranges between 4% and 60% of AMI causes, depending on the case-mix[9,10].

Several other smaller cohorts also reported hemodialysis as a setting associated with a risk of NOMI[11]. In a retrospective study of 57 cases occurring in the first 12 h after the last hemodialysis session, all cases were preceded by an episode of hypotension during hemodialysis, and investigations found diffuse (≥ 3) ischemic areas, in 20% of cases[12]. Vasculitis was also reported as an occasional cause of NOMI, especially polyarteritis nodosa[13].

In the ICU, while described for decades, interest in NOMI is growing; as shown by an increase in reporting in the last few years[2,3,14,15]. To date, the largest retrospective multicenter study gathered 780 AMI diagnoses in ICU patients, reporting an in-ICU mortality of 58%[15]. Of note, the occlusive or non-occlusive origin of AMI was not investigated. When AMI occurs in the ICU, NOMI appears prevalent: 91% of cases in a study of 101 AMI patients, with similar rates in other cohorts[4,16]. This increased prevalence in ICU may be explained by the fact that many conditions leading to ICU admission may be associated with a NOMI onset.

Several studies reported NOMI as a complication of cardiopulmonary bypass surgery, occurring in less than 1% of patients, often in patients with peripheral artery disease[4,17-19]. As a result, NOMI should be suspected in patients suffering from multiple organ failure after cardiac surgery; as suggested by Guillaume *et al*[4] in a cohort study of 320 patients in which NOMI rate was 10%[4]. In this study, the incidence of NOMI was not immediate: The authors reported a median of 7 d between cardiac surgery and NOMI diagnosis.

NOMI may also occur in patients admitted for successfully resuscitated cardiac arrest[5,20]. According to a recent report of a cardiac arrest center, NOMI may affect 2.5% to 6% of patients after cardiac arrest, mortality being 96%[5]. Factors reflecting the severity of the ischemia-reperfusion syndrome, such as higher admission lactate, low flow > 17 min, and higher inotropic score, were associated with NOMI diagnosis. Furthermore, NOMI represents a cause of secondary worsening in septic shock. Investigating the cause of death in septic shock according to time since ICU admission, Daviaud *et al*[21] identified NOMI respectively as the second and third causes of early (≤ 3 d) and late (> 3 d) death.

Mechanisms of AMI

The pathophysiological mechanical concept of AMI relies on an imbalance between oxygen supply and demand of the intestinal tissues. Ischemic lesions first begin to appear in the intestinal mucosa and subsequently may progress to irreversible transmural necrosis[22,23]. Complications include intestinal perforation, peritonitis, bacteriemia due to rupture of the gut barrier, inflammation leading to further non-mesenteric organ dysfunction, and shock. An essential contribution to the field was the historic work from Chiu *et al*[24] demonstrating how decreased mesenteric flow generates mucosal lesions. In an animal model, superior mesenteric artery blood flow was modulated, serial biopsies of the small intestine were performed, and ischemic intestinal mucosal lesions were detailed. The authors observed two observations of high importance. First, mucosal lesions appeared very early after the start of the experience. Second, the rapidity and the severity of mucosal lesions were correlated with the importance of decrease in blood flow. This experience highlights how urgent it is to make the diagnosis of AMI and proceed to treatment since vital and functional complications evolve quickly. A parallel can be drawn with acute myocardial infarction and stroke. Hence, intestinal stroke centers allowing early multimodal management have been suggested, and first reports showed increased survival[25].

Although the experiments performed by Chiu *et al*[24] strongly support the hypothesis of a supply-demand imbalance as a primary step towards NOMI, other complex processes may be involved, ultimately leading to the progression towards intestinal necrosis. These processes include the promotion of remote multiorgan failure through complex inflammatory pathways after a first insult in the form of transient hypoperfusion of the main mesenteric arteries[3,22,26]. Other mechanisms include impaired tissue perfusion responsible for gut barrier failure and endotoxin translocation, endothelial dysfunction and ischemia-reperfusion injury with increased local cytokine production, which may vary according to the primary cause of intestinal hypoxia[3,27-29].

In septic shock, tissue perfusion may be altered at the microcirculation level; despite seemingly optimized global hemodynamic parameters and these microcirculatory abnormalities are directly linked to organ failure[30]. Notably, Dubin *et al*[31]

demonstrated persistence of altered intestinal microcirculation disorders in deceased animals after correction of arterial hypotension in a model of septic shock[31]. Moreover, in sepsis, other mechanisms may participate to tissue dysoxia: Cellular and metabolic disorders[32,33]. Lobo *et al*[34] showed possible "cytopathic hypoxia" without impairment of oxygen delivery in the development of gut mucosal injury during endotoxic shock[34]. Therefore, a primary transient main mesenteric arteries hypoperfusion may not be a mandatory step in NOMI related to sepsis.

Although NOMI is thought to represent the worst stage of acute gastrointestinal injury in critically ill patients[35], the exact pathophysiology is still poorly understood, and the definition of this concept remains unclear[36]. According to a working group of the European Society of Intensive Medicine, NOMI is one of the possible facets of acute gastrointestinal dysfunction. In a recent update, acute gastrointestinal dysfunction is outlined as the consequence of a multitude of interacting pathophysiological mechanisms, resulting in other life-threating conditions such as Ogilvie's syndrome, sepsis, gastrointestinal tract perforation or bleeding, and acute compartment syndrome[36].

Additionally, deleterious therapeutic interventions may add to the incidence of NOMI by worsening tissue dysoxia in ICU patients. Experimental and observational studies suggest that the use of vasopressors such as norepinephrine and epinephrine might result in impaired mucosal perfusion[37-39]. Other pharmacological agents such as vasopressin and digoxin[3] as well as acute profound hypovolemia could also worsen ischemic lesions. Lastly, the role of enteral nutrition in critically ill patients is controversial and depends on several factors such as the dose of enteral nutrition, the metabolic phase, and the severity of the patients. In the recent randomized controlled trial "NUTRIREA 2"[40], enteral nutrition was compared to parenteral nutrition with a normocaloric target (*i.e.* 20-25 kcal/kg *per* day) during the first days of admission (*i.e.* catabolic phase) in mechanically ventilated patients with shock. Mortality did not differ between the two groups, but a significantly higher rate of bowel ischemia was reported in the enteral group [19 (2%) patients *vs* 5 (< 1%) patients]. However, an ancillary study focused on citrulline and intestinal-fatty acid binding protein (I-FABP) biomarkers showed possible protective effects of enteral nutrition on enterocyte mass, raising an interest for further investigation[41]. In particular, some hypothesized that a lower dose of enteral nutrition in these patients may yield a protective effect[42].

Compared with occlusive AMI, NOMI reported mortality is higher, ranging between 70% and 100% depending on the series[4,5,16]. While complications of NOMI are similar to those of occlusive AMI (including necrosis, perforation with peritonitis, bacteremia secondary to digestive translocations, acute compartment syndrome, vasoplegic shock, multi organ failure leading to death), their prognosis is indeed different[2]. In survivor patients, late AMI complications classically include short bowel syndrome, undernourishment, and need of total parental nutrition[43,44]. Of note, late outcomes in NOMI patients (*e.g.*, long-term mortality, quality of life) are currently unknown and should be investigated.

Several reasons may explain this poor prognosis in NOMI as compared to occlusive AMI. To start, in the former, patients are in a critically state, due to an earlier severe aggression, and NOMI represents a "second-hit" added on top of the reason for ICU admission. Secondly, treatment options do not allow a rapid reversal of the causal insult (as opposed to a revascularization of an occluded vessel), leading to late treatment and thus worse outcomes. Thirdly, surgical treatment is complex because of the lack of clear delimitation between viable and necrotic tissue: Lesions are often diffuse or patchy and extensive resections are then performed, when deemed relevant, which often is not the case after laparoscopic evaluation. Lastly, diagnosis is complex and requires multimodal approaches, leading to delays, as compared to obstructive AMI causes[3,45].

DIAGNOSTIC PITFALLS AND CHALLENGES FOR FUTURE RESEARCH

Facing a rapidly evolving disorder, the diagnostic process should provide answers to two important issues. First of all, diagnosing AMI early is essential to avoid progression to transmural necrosis and the associated complications[3,46]. Secondly, reliable information on the presence or absence of intestinal necrosis, and ideally on the intestinal location involved, is needed to guide decision regarding surgical treatment, according to the location and indication of surgery and patients' condition. The importance of this issue is summarized in Figure 1.

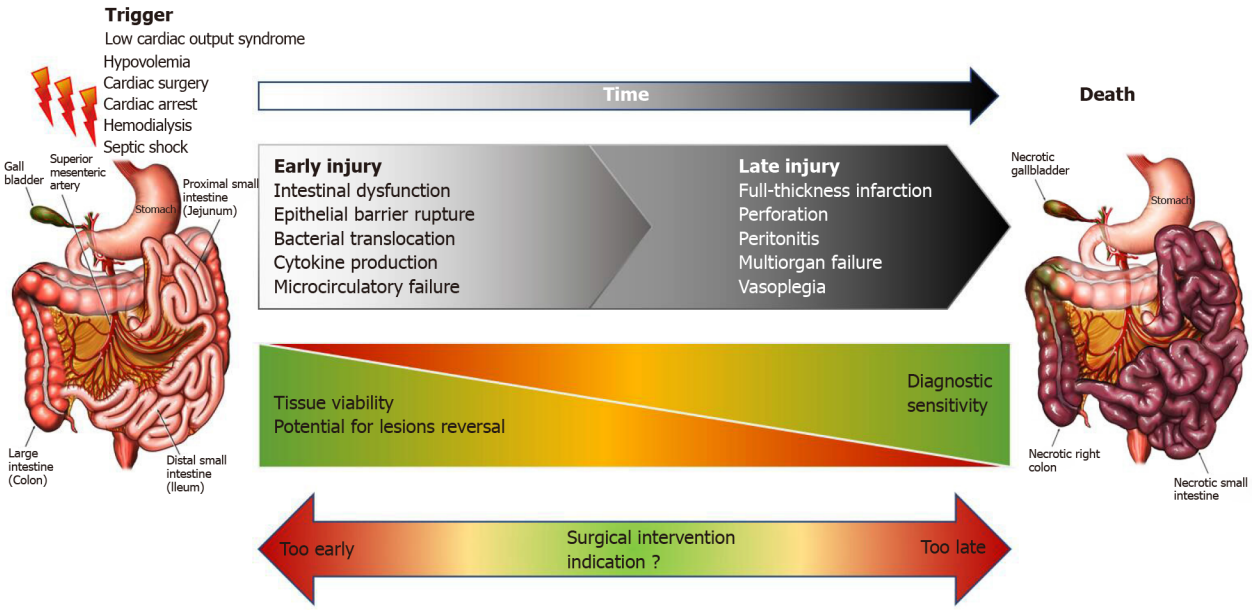


Figure 1 Timing of events in non-occlusive mesenteric ischemia.

As of today, these vital questions remain unanswered in the setting of NOMI and are often diagnosed too late. At time of diagnosis in NOMI studies, severity biological parameters are usually markedly high, and necrosis is frequently observed in comparison to recent occlusive AMI cohorts[4,5,16,47]. This severity, possibly associated with diagnostic delay, could partially explain the discrepancies between observed mortality rates.

Clinical examination

NOMI has always been presented as a challenging diagnosis due in particular to clinical signs considered to be non-specific[45,46]. Diagnosis is usually suspected in front of novel or worsening of circulatory failure in evocative contexts (*e.g.*, cardiac surgery or after cardiac resuscitation) and is discussed as a differential diagnosis of secondary infections[3]. Digestive signs, similar to that of an occlusive AMI presentation, might then evoke NOMI. These signs include possibly "brutal" abdominal pain, diarrhea, upper or lower digestive hemorrhage, and vomiting. However, a notable difference is that the beginning of occlusive AMI is often brutal allowing to pinpoint the exact onset time; in contrast, in the ICU setting, patients may be sedated and curarized, abdominal exam may not yield much, even if other non-specific digestive signs might suggest NOMI (abnormal gastric residual volume under enteral nutrition, ileus, increase of abdominal perimeter, increased intra-abdominal pressure).

Biomarkers

Biomarkers represent additive tools to this difficult diagnosis. Routine biomarkers, among those reflecting tissue ischemia, plasma lactate, lactate dehydrogenase (LDH), and aspartate aminotransferase (AST), can reinforce NOMI suspicion[45,48].

D-dimers have also been reported as highly sensitive for AMI diagnosis with plausible negative predictive value[49-51]. However, no study focused on a selected NOMI population in which it is likely to find multiple concurring causes for an increase in D-dimer levels, resulting in low specificity, and its dosage is not recommended[2]. Similarly, this conclusion can be drawn with LDH levels and leukocyte count[16,52,53].

After cardiac surgery, there has been interest in AST[53,54]. A value ≥ 100 IU/L was independently associated with AMI diagnosis in patients with multi organ failure[4]. Interestingly, AST was also associated with mortality in 780 AMI patients from various causes[15]. Thus, despite obvious lack of specificity, elevation of AST may reinforce clinical suspicion.

Procalcitonin has also been shown to be associated with mortality in AMI patients [55]. A threshold value of 2.47 ng/mL was suggested in a monocentric retrospective study of 128 AMI patients[56]. However, in a NOMI subgroup, procalcitonin may be less accurate given the high prevalence of acute renal failure and infections[57].

Serum lactate, a long-time marker of tissue ischemia, is usually associated with mortality in the AMI setting[15]. Despite a lack of specificity, lactate could be useful to predict necrosis when associated with other parameters. A prospective study of 67 selected patients with AMI identified three parameters associated with necrosis: Presence of organ failure, serum lactate levels > 2 mmol/L, and bowel dilation on computed tomography (CT) imaging[47]. When all three parameters were present, necrosis requiring surgical resection was highly likely. However, there was only one patient with NOMI in this cohort, and, in the setting of NOMI, an increase in plasma lactate levels is consistent with numerous possible etiologies.

Yet, clinical exam and routine laboratory tests are of only little value to make an early reliable diagnosis and to differentiate suspicion from confirmed NOMI[1,16,58].

Perspectives in biomarkers

Research is in progress to identify candidate AMI biomarkers. One of the most promising is I-FABP. Preliminary studies suggest a potential interest in I-FABP, a small cytosolic protein specific to small bowel released in the context of intestinal ischemia[59]. Experimental studies demonstrated early increase of I-FABP after onset of gut ischemia[60]. Thuijls *et al*[52] studied plasma and urinary I-FABP accuracy in 46 patients with a suspicion of AMI, of which 22 AMI cases were finally confirmed[52]. The area under the receiver-operating curve (AUC) for urinary I-FABP was 0.93, performing better than plasma I-FABP (AUC = 0.70). Notably, the increase in I-FABP was greater in patients with ischemia of the ileum, which is the main source of I-FABP production. However, in critical illness and particularly in NOMI, acute renal failure is highly prevalent, and urine samples might not be available. Further studies are needed to refine plasma I-FABP accuracy.

Interestingly, Matsumoto *et al*[53] found an AUC of 0.88 for AMI diagnosis including 15 cases of NOMI and 9 arterial occlusions[53]. The authors also highlighted that I-FABP is increased in various non-vascular intestinal ischemia etiologies such strangulated bowel obstruction, incarcerated hernia, and volvulus. While promising, the integration of plasma I-FABP to the routine monitoring of intestinal ischemia is probably too early at this point and should be further explored. In adults with septic shock, Sekino *et al*[61] measured daily plasma I-FABP in a monocentric observational study and found a higher incidence of NOMI when I-FABP levels were superior to a threshold of 19.0 ng/mL[61]. Importantly, I-FABP thresholds for AMI diagnosis are not consensual[29], and differences in accuracy of I-FABP dosage according to enzyme-linked immunosorbent assay kits lead to further limitations[62].

Plasmatic citrulline, an amino acid synthesized from glutamine by small bowel enterocytes and metabolized into arginine by the kidney, reflects functional enterocyte mass and has been proposed as a marker of acute intestinal failure in critically ill patients[63]. However, the high prevalence of acute renal failure in the ICU population may lead to high plasma citrulline concentrations despite a reduction of enterocyte mass[64]. Further studies are needed to precise its performance in critical illness and NOMI diagnosis. To a lesser extent, the ability of endothelin-1 to predict NOMI has been investigated in 78 post cardiac surgery patients and revealed high specificity (94%) but poor sensitivity (51%)[65].

Imaging

From clinical suspicion of NOMI to certitude, diagnosis relies on imaging. Historically, angiography was considered pivotal by some experts, as it was considered an efficient treatment for NOMI[66,67]. Angiographic observations of NOMI included the visualization of absence of large artery occlusion and vasoconstriction of small intestinal arteries. Subsequently, angiography enabled the *in situ* administration of a continuous infusion of vasodilatory drugs like papaverine. Small cohort studies reported efficacy, suggesting this treatment may be associated with fewer progression to necrosis, and improved survival[26]; the effectiveness of this strategy may not be warranted if NOMI is diagnosed at the stage of intestinal necrosis requiring surgical treatment. Moreover, tolerance of vasodilatory drugs in hemodynamically unstable patients is unclear, and given the low availability of the technique, it remains reserved for expert centers. Hence, evidence diagnostic and therapeutic angiography interventions in NOMI remain low, for now.

On the other hand, while contrast-enhanced abdominal CT scan plays a central role in occlusive AMI[68], indicating the occluded vessel and eventually guiding revascularization possibilities, its performance in NOMI is disappointing. A monocentric study compared the classical CT signs evoking AMI of 75 patients with NOMI, with 39 patients in which NOMI was suspected but subsequently ruled out, when compared to macroscopic diagnosis considered as reference[16]. Portal venous gas, pneumatosis

intestinalis, and abnormal contrast-induced bowel wall enhancement exhibited good specificities (respectively 95%, 85%, and 71%) but were poorly sensitive to the point, that one quarter of patients exhibited mesenteric ischemia without any suggestive radiological signs.

Abdominal ultrasound has been recently proposed for the investigation of acute gastrointestinal injury, emphasizing the possibilities to measure gastrointestinal diameter, mucosal thickness, peristalsis, and blood flow[69]. As of today, data on ultrasound performance for NOMI diagnosis are scarce, despite the evident advantage of being performed at the bedside non-invasively and the ability to diagnose bowel dilation, intramural, or portal venous gas[70,71].

Endoscopy

Finally, endoscopy is widely used in the ICU setting and presents the advantages of direct visualization of intestinal mucosa at the bedside. Given the relatively low negative predictive value of CT imaging, endoscopy is frequently performed and allows to diagnose a significant number of NOMI cases in the ICU. In post cardiac arrest patients, hemorrhagic or necrotic lesions are likely to be found during gut endoscopy in the presence of clinical signs of gastrointestinal dysfunction[72]. However, its disadvantages are numerous: A large part of the intestines (*i.e.* small bowel) are inaccessible, the observed mucosal necrosis does not always correspond to transmural necrosis, there exists an inherent risk of perforation in weakened tissues, and availability is dependent on the operators.

Hence, given the numerous pitfalls of the current diagnostic approach for the diagnosis of NOMI, a high index of suspicion is required in populations at risk, such as post cardiac or aortic surgery, hemodialyzed patients, and critically ill patients[23,73]. Research is encouraged to identify or validate new biomarkers and imagery tools and increase knowledge on the pathophysiological understanding of NOMI genesis, especially in critically ill patients[36]. Specific accuracy of these new biomarkers should be further evaluated in the future. However, well designed studies are incredibly difficult due to numerous issues. Importantly, the low incidence of NOMI requires an appropriate selection of the study population with consideration of the pre-test probability. Additionally, patients in which NOMI has been ruled out are difficult to define given the low negative predictive value of CT imaging. Methodological difficulties originate from the lack of knowledge of the physiopathology and the important variability of NOMI time course due to differences in the intensity and duration of the aggression at the origin of NOMI. Furthermore, a working group of the European Society of Intensive Care Medicine stated the need for a consensus definition of NOMI in order to improve the current knowledge, study epidemiology and suggest interventions[36].

Overview of AI in healthcare

AI is a vague term reflecting the use of computers to perform tasks that are thought to require unique skills, often in ways that are hard to pin-point and that evolve with time. For example, although basic game algorithms such as those initially developed for chess, were considered as such 50 years ago, they are now part of every personal computer, and we know how to break them down into discrete steps and feel we understand them[74].

Later, AI encompassed the field of image recognition[75]. Although we humans perform this task naturally, we often cannot articulate exactly how this is done. This lack of supervision is one of the features of machine learning (ML), a subset of AI. It is the study of algorithms that learn from experience without being explicitly programmed for their task. ML incorporates a broad range of statistical methods ranging from linear regression to support vector machines, decision trees, or neural networks that make use of new datapoints to update the function they approximate [76].

As introduced, this field is subdivided into supervised methods, which learn from labeled samples, and unsupervised methods that attempt to find patterns in the data themselves[76,77]. The main applications of supervised models are classification, in other words, choosing to which predefined class an observation belongs, and regression, in which a value is derived from given observations. In medical imagery, these two applications often amount to diagnosis (classification) and prognostication (regression). Clustering, in which observations are grouped in classes that are not predefined, and dimensionality reduction used for data structuring or visualization are the most common applications of unsupervised learning. Finally, in reinforcement learning, a model interacts with its environment, performs actions, and learns in a trial-and-error fashion. The main applications of reinforcement learning lie in decision

support tools and autonomous agents[78].

During the development of a ML system, the parameters of a model, termed weights, are gradually adjusted to fit a training dataset[79]. In a second step, the model is validated on a separate dataset. An evaluation on the initial dataset would result in overly optimistic results, dubbed the overfitting effect. This common phenomenon occurs when the ML model adapts itself too much to the training dataset and then fails to generalize on other datasets. In other terms, the model remembers the examples seen in the training phase but does not learn any relevant features that are applicable to future observations.

The simplest way to derive a model from a set of observations is variable thresholding. When combining multiple features, linear and logistic regression are the most frequently used techniques in healthcare but require the assumption of normality [80]. Methods capable of using non-linear discriminant functions such as support vector machines as well as methods relying on multiple linear boundaries such as decisions trees have been elaborated. While simple to implement, these models are limited in their ability to process raw data, such as images or time course data, as they struggle to model the relationships of large amounts of variables in multiple dimensions[81]. Instead, the elaboration of such models often needs to rely on considerable domain expertise to extract relevant traits from the raw data, yet, arbitrarily selected features and the underlying physiologic assumptions may fail to capture a specific individual's response. This is especially true for the analysis of medical images, in which every voxel represents an individual variable influenced by location, tissue type, and surrounding structures as well as time-sensitive data such as those recorded in standardized electronic healthcare records (EHRs).

Specific ML algorithms that are built in a multi-layered fashion, termed deep learning (DL) algorithms, circumvent this limitation by automatically encoding multiple levels of inner representations of relevant features. This is achieved by composing simple non-linear units that sequentially transform the representation, starting from the raw data, into a slightly more abstract representation at a deeper level. Visually, when analyzing images, the first two layers often represent edges and particular arrangements of edges. Subsequent deeper layers then assemble the motifs encoded in the prior layers into larger combinations representing parts of patterns featured in the raw data. The main advantage of this process is that the features are learnt by a general-purpose learning algorithm without any direct human intervention. This allows for the rapid development of models able to discover intricate features in multi-dimensional data. In the last decade, DL has led to major improvements in performance in the fields of computer vision[75,82] and natural language processing[83,84]. The main model architectures used in these domains are convolutional neural networks, recurrent neural networks (RNNs), auto-encoders, and transformers. Even if DL methods have been able to produce spectacular results, it is important to realize that these methods are still in their early days, and their performance does not always exceed that of conventional techniques using hand-selected features[85]. DL works well with large datasets but often requires specific computational infrastructure for the training process, whereas conventional ML methods have advantages for smaller datasets and can be created with classical processors.

The advantages brought by DL-powered data analyses have rapidly been taken over into the medical domain with first translations to radiology[86], ophthalmology [87] and pathology. Although the implementation of such algorithms in a clinical setting remains challenging[74], this progression has culminated in the approval of the first insurance reimbursement for AI augmented medical care[88] for the CT-based detection of large vessel occlusion in stroke. Modern ICUs generate vast streams of data stored in EHRs and current in-silico research has yielded successful DL tools to improve the prediction of mortality[89-93] and to guide clinical decisions[94]. A major focus has been the prediction of sepsis, which, analogously to NOMI, lacks a distinctive marker for an accurate and timely diagnosis. In recent years, multiple ML methods have emerged to diagnose sepsis in real-time or to predict its occurrence. The most prominent models relied on RNNs[95,96], custom hazard models[97] or a combination of multiple models, known as ensembles. Although clinical validation studies are often still lacking, these automated methods offer new possibilities for the early detection of sepsis based on objective variables extracted from EHR data[98,99]. Similarly, the lack of a gold standard non-invasive definition of NOMI and the need for rapid detection make for an excellent opportunity for the application of ML.

Diagnostic approaches in NOMI

Likewise, the diagnosis and management of NOMI highly depends on information obtained from imaging studies, clinical variables, and biological findings. Yet, no single marker allows for the accurate detection of intestinal ischemia. The expertise of gastroenterologists, intensivists, radiologists, and surgeons remains mandatory, but their availability and the time needed to process all these complex data may delay timely surgical intervention.

In NOMI prediction, multivariate logistic regressions models have been described several times[16,100-102]. When applying a threshold to a linear combination of weighted clinical variables, these have been used for the prediction of NOMI in 865 patients after cardiac surgery, of which 78 were angiographically confirmed to have developed mesenteric ischemia[19]. According to the authors, this linear discriminant analysis yielded a sensitivity of 76.9% and specificity of 93.8%. The interpretation of these results remains, however, limited as variables and weights were derived and tested in the same cohort. A follow-up logistic regression model used preoperative, intraoperative, and postoperative risk factors derived from 4449 patients after cardiac surgery to predict the occurrence of NOMI[103]. The authors report an AUC of 0.91 in their control cohort (n = 4299). Although these are encouraging results, the evaluation of these models suffers from methodological flaws as derivation and validation datasets of the model weights were not distinct.

Future models may benefit from more advanced algorithms such as those employed for the prediction of sepsis as discussed above. Furthermore, using continuous data streams instead of single timepoints as input would result in models with closer resemblance to clinical and physiologic reality. Long short-term memory networks in particular, a specific form of RNN, have shown promising results on temporal sequences sampled from ICU EHRs[93,95,96]. The so-called transformer, a successor model to long short-term memory networks integrating the concept of selective attention, has since emerged from the natural language processing domain[104]. Although the application of transformer models to medical EHRs is only beginning [105,106], it is possible model architecture will be prominent in the coming years.

Abdominal CT findings can reveal intestinal ischemia, although inter-rater agreement often remains limited. A multivariate combination of radiological signs has been identified through a logistic regression model in a cohort of 68 patients requiring cardiopulmonary bypass during surgery[107]. The resulting model was not accurately validated but performed well on the training cohort (AUC = 0.84). A model for the detection of transmural intestinal infarction confirmed on laparotomy has been elaborated on CT scans of 207 patients with superior mesenteric venous thrombosis [108]. A follow-up validation on an external cohort (n = 89), demonstrated satisfying performance (AUC = 0.84) and led to the development of a nomogram. Although this model has been developed in a different patient population, it remains one of the most accurately validated models for the detection of intestinal ischemia.

The use of image-based models could strongly improve performance and usability as they do not depend on the detection of a few selected findings and may integrate holistic imaging features, using convolutional neural networks such as those used in abdominal CT scans for the detection of acute appendicitis[109,110]. It is of note that although feature-based models (such as presence of pneumatosis intestinalis or abnormal bowel wall enhancement) for the detection of intestinal ischemia developed on patients with occlusion may translate to patients without occlusion, the features used by image-based models are often hidden to the user and will inadvertently rely on findings extracted from the site of occlusion. Much attention should therefore be paid to a careful selection of the study population and a clear restriction of use-cases for the developed models.

Perspectives in NOMI prediction

The availability of diverse and complex data points makes the use of AI for the detection and prognostication of NOMI in the ICU a valuable clinical opportunity. Currently developed ML models show encouraging results but lack rigorous statistical validation. Moreover, the use of state-of-the-art DL methods is likely to benefit model performance. As such, algorithms can encode an inner representation of relevant patterns and can approximate more complex non-linear functions, their use would forego the need of handcrafted features. This is especially relevant for the analysis of temporal sequences extracted from EHRs and abdominal CT scans. Indeed, although many signs of bowel ischemia have been identified, their performance[16] and inter-rater agreement remain limited[107,111]. Recent advances in fusion models leveraging both imaging and EHR data have performed well in the detection of pulmonary

embolism[112] and may be tested in NOMI. To fast-forward the development of future models there is a clear need for the collection and release of datasets incorporating patients with suspicion and definite diagnosis of NOMI. The diagnosis of NOMI in such datasets should ideally be verified pathologically or surgically, be it *via* laparotomy or laparoscopy, to obtain a clean target definition for model development. Moreover, it is essential to obtain clinical validation through prospective studies of not only the performance of developed models but also the ease of implementation into the ICU setting and their clinical utility following recently published guidelines[113, 114]. While most attention is often directed to increasing predictive performance, future AI solutions should account for their predictions to lead to wider clinical applicability and acceptance. Ideally, future AI systems should therefore strive to achieve ease-of-use, interpretability, and diagnostic performance.

It is likely that the results achieved by ML will continue to improve as the computational power at disposition increases, collected datasets grow, and more performant and adequate algorithms are developed. When applied to EHR data and medical imaging with statistical rigor, ML models could refine the accuracy and speed of diagnosis of NOMI in critically ill patients. Used appropriately, this emerging technology could further be leveraged to identify and explore new disease mechanisms and single-out yet unrealized connections between datapoints, paving the way for a deeper understanding of the intricated interactions leading to NOMI in the ICU.

CONCLUSION

NOMI is associated with poor prognosis due to lack of accurate diagnostic tools. While taken individually, several biomarkers and imagery modalities exist, their combination and the study of their variation through time, which requires sheer computational power that may be provided by artificial intelligent tools, is bound to increase diagnostic performance in NOMI and improve therapeutic management.

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REVIEW

Hepatocellular carcinoma in patients with renal dysfunction: Pathophysiology, prognosis, and treatment challenges

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Abstract

The population of patients with hepatocellular carcinoma (HCC) overlaps to a high degree with those for chronic kidney disease (CKD) and end-stage renal disease (ESRD). The degrees of renal dysfunction vary, from the various stages of CKD to dialysis-dependent ESRD, which often affects the prognosis and treatment choice of patients with HCC. In addition, renal dysfunction makes treatment more difficult and may negatively affect treatment outcomes. This study summarized the possible causes of the high comorbidity of HCC and renal dysfunction. The possible mechanisms of CKD causing HCC involve uremia itself, long-term dialysis status, immunosuppressive agents for postrenal transplant status, and miscellaneous factors such as hormone alterations and dysbiosis. The possible mechanisms of HCC affecting renal function include direct tumor invasion and hepatorenal syndrome. Finally, we categorized the risk factors that could lead to both HCC and CKD into four categories: Environmental toxins, viral hepatitis, metabolic syndrome, and vasoactive factors. Both CKD and ESRD have been reported to negatively affect HCC prognosis, but more research is warranted to confirm this. Furthermore, ESRD status itself ought not to prevent patients receiving aggressive treatments. This study then adopted the well-known Barcelona Clinic Liver Cancer guidelines as a framework to discuss the indicators for each stage of HCC treatment, treatment-related adverse renal effects, and concerns that are specific to patients with pre-existing renal dysfunction when undergoing aggressive treatments against CKD and ESRD. Such aggressive treatments include liver resection, simultaneous liver kidney transplantation, radiofrequency ablation, and transarterial chemoembolization. Finally, focusing on patients unable to receive active treatment, this study compiled information on the latest systemic pharmacological therapies, including targeted and immunotherapeutic drugs. Based on available clinical studies and Food and Drug Administration labels, this study details the drug indications, side effects, and

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dose adjustments for patients with renal dysfunction. It also provides a comprehensive review of information on HCC patients with renal dysfunction from disease onset to treatment.

Key Words: Hepatocellular carcinoma; Chronic kidney disease; End-stage renal disease; Hemodialysis; Cancer prognosis; Cancer therapeutics

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Core Tip: The varying degrees of renal dysfunction, from the various stages of chronic kidney disease to dialysis-dependent end-stage renal disease, often affect the choice of treatment and prognosis of patients with hepatocellular carcinoma (HCC). This complicates HCC treatment. This review encompasses the presumptive causes of the high degree of comorbidity of HCC and renal dysfunction, the impact of renal dysfunction on HCC prognosis, and the concerns that are specific to patients with pre-existing renal dysfunction for each stage of HCC treatment.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a common malignancy worldwide and accounts for substantial morbidity and mortality[1,2]. However, the etiology, incidence, and mortality of HCC are geographically uneven. Most HCC cases are found in East Asia and Sub-Saharan Africa. Although the incidence rates are relatively lower in Western countries, the mortality rates remain high[1]. The major risk factors for HCC include chronic hepatitis B virus (HBV), chronic hepatitis C virus (HCV), cirrhosis, alcoholic liver disease, and nonalcoholic steatohepatitis (NASH)[3]. Another leading cause of morbidity and mortality worldwide is renal dysfunction, which includes chronic kidney disease (CKD) and end-stage renal disease (ESRD). Caring for patients with renal diseases has greatly burdened health care systems[4]. Notably, patients with renal dysfunction have a higher prevalence of cancer, including liver cancer, compared with the general population[5,6]. Furthermore, such patients—especially those on maintenance dialysis for ESRD—were reported to have a higher prevalence of viral hepatitis compared with the general population[7]. In certain regions where both renal dysfunction and HCC are highly prevalent, the two conditions are highly comorbid [8]. In the present study, we searched and organized the available evidence-based literature to provide a comprehensive review guided by the following research questions: (1) Does any correlation or causality exist between renal dysfunction and the development of HCC? (2) Would renal dysfunction, including CKD and ESRD status, affect the prognosis and treatment outcomes of HCC? And (3) What are the challenges of treating HCC in patients with renal dysfunction in all categories of the Barcelona Clinic Liver Cancer (BCLC) system algorithm?

Despite the lack of a validated international consensus on the management of patients with both renal dysfunction and HCC, we aimed to summarize information that is critical to the development of preventive and therapeutic strategies for this specific population.

ASSOCIATION AND POSSIBLE PATHOPHYSIOLOGICAL MECHANISMS BETWEEN RENAL DYSFUNCTION AND HCC

A high incidence of cancer has been reported in patients with renal dysfunction. Both CKD and ESRD were reported to be bidirectionally connected to cancer: Renal

dysfunction can serve as a risk factor for cancers including HCC, whereas cancer and related treatments can directly or indirectly lead to or aggravate renal dysfunction[6]. In addition to the mutual relationship between renal dysfunction and cancer, renal dysfunction and HCC share common risk factors that complicate the association between the two diseases. These risk factors can be categorized into vasoactive factors and those related to environmental toxins, viral hepatitis, and metabolic diseases (Table 1). The complex pathways linking renal dysfunction and HCC are depicted in Figure 1. The following subsections summarize the studies that provide evidence for each of these links.

Renal dysfunction as a cause of or risk factor for HCC

Cancer risk has been reported to be elevated in patients with renal dysfunction. Numerous studies have demonstrated an increased risk of liver cancer in patients with ESRD on dialysis[9-11]. Limited data are available on whether less advanced CKD, where dialysis is not needed, can increase the risk of liver cancer[12,13]. Several hypotheses have been proposed for these correlations, including a dysregulated immune system, defective DNA repair mechanism, impaired antioxidant defense, accumulation of carcinogenic compounds caused by reduced renal elimination, and the uremia milieu[14]. Because it is well-established that kidney transplant recipients have an increased cancer risk because of the aggressive administration of immunosuppressive agents[15], it is reasonable to have a separate discussion for post-kidney-transplantation status.

CKD without dialysis − accumulation of uremic toxins and elevated serum levels of cytokines: The well-documented phenomenon of patients with impaired renal function having increased cancer risk raises the following question: Could uremic toxin accumulation, one of the direct consequences of renal dysfunction, be carcinogenic?

P-Cresyl sulfate (PCS), a protein-bound uremic toxin prototype that cannot be efficiently removed through routine dialysis procedures, has been found to be fibrogenic in the kidney and vascular system of mice through epithelial-to-mesenchymal transition (EMT)[16-18]. EMT is an irreversible process through which epithelial cells lose their cell polarity and cell–cell adhesion and acquire migratory properties to become mesenchymal cells. EMT has also been implicated in the development of liver fibrosis and cirrhosis[19-22]. It has been widely accepted that transforming growth factor-beta (TGF-β) plays a crucial role in hepatic EMT through stellate cell activation and excessive matrix synthesis[19,20,23,24]. The sequential progression from chronic liver fibrosis to cirrhosis culminates in the development of HCC, which is a major cause of death in patients with compensated cirrhosis[25,26]. A study in Taiwan found that PCS increased the incidence of liver fibrosis in people with HBV and HCV[27]. Although limited data exist on whether the PCS can directly induce EMT in the liver, we postulate that PCS accumulation secondary to renal dysfunction influences liver fibrosis, cirrhosis, and eventually the risk of HCC. Hwang *et al*[28] conducted a population-based study to examine the mechanism behind the high incidence of HCC in ESRD, and they found PCS to be positively correlated with HCC occurrence[28]. However, in that study, ESRD was no longer associated with a higher incidence of HCC than in the general population after matching was conducted for hepatitis and liver cirrhosis. Those authors concluded that the high incidence of HCC in patients with ESRD was caused by a high viral hepatitis rate rather than by uremia *per se*[28]. This does not violate our aforementioned assumption, despite appearing to do so, that PCS indirectly contributes to HCC occurrence through liver inflammation and cirrhosis. Further studies are warranted to clarify the link between PCS and HCC.

Cytokines constitute another topic worthy of discussion. Renal dysfunction is also known to increase the level of cytokines in the body, such as interleukin (IL)-1, IL-6, and tumor necrosis factor-α (TNF-α)[29-32]. Several studies have reported that IL-6, TNF-α, and other cytokines are associated with more severe problems in liver necrosis, tissue repair and regeneration, and the accumulation of mutations caused by aberrant cell proliferation, thus increasing the transformation potential of hepatocytes and the risk of HCC[33,34]. More studies focusing on the underlying pathophysiology are required to determine whether these cytokines lead to hepatic carcinogenesis, either directly or indirectly. In addition to proinflammatory properties, uremia has been recognized to compromise normal immune response by enhancing the apoptosis of activated immune cells[35-37]. The immune alterations associated with uremia possibly contribute to cancer occurrence. However, data on site-specific cancers such as HCC are lacking, and further studies are required to delineate the relationships

Table 1 Shared risk factors for hepatocellular carcinoma and chronic kidney disease

Risk factors	
Environmental toxins	Arsenic
	Cadmium
	Aflatoxin
	Aristolochic acid
Viral hepatitis	Hepatitis B virus
	Hepatitis C virus
Metabolic syndrome and related disorders	Non-alcoholic fatty liver disease
	Nonalcoholic steatohepatitis
	Diabetes mellitus
Vasoactive factors	Renin-angiotensin system activation

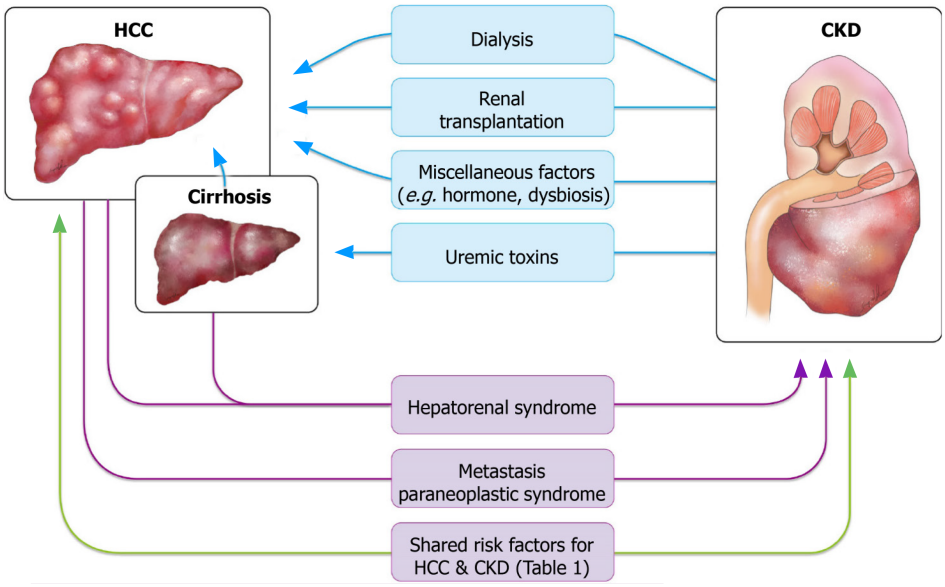


Figure 1 Association between hepatocellular carcinoma and chronic kidney disease. This figure summarizes the confirmed and presumptive links between hepatocellular carcinoma and renal dysfunction. HCC: Hepatocellular carcinoma; CKD: Chronic kidney disease.

between uremia, immune dysfunction, and HCC.

Effect of ESRD on chronic dialysis status: Studies have suggested that the overall cancer risk in chronic dialysis patients is significantly higher than that in the general population, for both HCC and cancers of other primary sites[10,12,13]. Whether such carcinogenic effects originate from the dialysis procedure itself or other ESRD-related factors remains to be determined[38]. Wong *et al*[39] found an increased dialysis time to be a significant risk factor for common solid organ cancers regardless of age. They demonstrated that a dose-dependent relationship exists between the duration of maintenance dialysis and overall cumulative cancer risk, and this relationship was independent of the dialysis modality. The findings were attributed to the immunodeficient and chronic inflammatory status in uremia and to the substances the patient was exposed to during dialysis, including nitrites, chloramines, and other unknown elements[39]. Another study on the pattern of excess cancer in dialysis and transplantation reported that dialysis was associated with a small increase in immune deficiency–related cancers, including liver cancer; however, the risk of liver cancer was not particularly high in patients on dialysis [standardized incidence ratio (SIR) 2.2, 95% confidence interval (CI): 1.2-3.7] relative to other types of immune deficiency–related cancers. Moreover, the overall findings for such cancers would be unchanged if liver cancer was excluded from the group[5]. Because both HBV and HCV are prevalent in patients on hemodialysis[40-42], studies have attributed HCC

incidence in part to exposure to oncogenic virus infection in dialysis populations through blood transfusion and contamination. Furthermore, a nationwide study in Taiwan revealed that liver cancer was the second most common cancer found in patients receiving dialysis and that the SIR of liver cancer in chronic dialysis patients was also higher than that of their healthy counterparts[9]. This result is inconsistent with the relatively lower frequency of liver cancer found in an international collaborative study[10]. A high frequency of liver cancer among the dialysis population might be explained by HBV and HCV infection being endemic to Taiwan[9]. In the aforementioned studies, except for the effects of infection-related factors, whether the dialysis procedure itself increases HCC risk remains inconclusive.

Kidney transplantation: Kidney transplantation is known to be associated with a marked increase in cancer risk at various sites[12]. In the late 1960s, the immunosuppressive agents administered to patients who underwent a transplant were discovered to increase the risk of cancer; compared with that in recipients of a cardiac or hepatic transplant, the aforementioned risk is a major outcome factor in recipients of a kidney transplant because of their longer survival owing to dialysis being widely available [43]. However, studies that have discussed HCC separately have reported mixed results on whether kidney transplant increases the incidence, reporting either no trend [5] or only a moderately increased risk[12,44]. Therefore, studies have provided limited support for the theory that kidney transplantation and the related application of immunosuppressive agents increases the risk of HCC.

Miscellaneous factors: HCC is more prevalent in men than in women. Both androgen and estrogen sex steroids can contribute to the gender disparity in HCC prevalence, where their effects are distinct to each sex[45]. Higher levels of androgen signaling are associated with an increased risk of HBV-related HCC[46,47], whereas higher estrogen pathway activity plays a protective role in female hepatocarcinogenesis. The estrogen axis is critical for maintaining a lower serum IL-6 level, thus reducing liver cancer risk in women[48,49]. A large cohort study[50] found CKD to increase liver cancer mortality in women and, to a lesser extent, in men; the gender disparity is likely explained by CKD-related hypogonadism, but this remains to be examined. The following sections discuss other possible risk factors shared by HCC and CKD, including environmental toxins, metabolic diseases, and genetic factors and their connections with CKD. However, the causal effects between these factors with CKD and HCC are still being debated.

Dysbiosis, which refers to the qualitative and quantitative alteration of gut microbiota, has been commonly observed in CKD patients. The imbalance of pathogenic flora and symbiotic flora was also implicated in the progression of CKD, increased cardiovascular risk, uremic toxicity, and inflammation[51]. An enhanced permeability of the intestinal barrier, allowing the passage of endotoxins and other bacterial products into the blood, was also reported in CKD[52]. Notably, dysbiosis is another possible risk factor for HCC that has been identified in recent years. Enterohepatic circulation is accompanied by low-grade exposure to gut microbiota–derived metabolites and products, often termed microbiota-associated molecular patterns (MAMPs)[53]. Changes in the intestinal barrier cause leakiness, leading to hepatic exposure to MAMPs. Accumulating evidence from the last decade, mostly from animal studies in rodents, suggests a key role of gut microbiota in the progression of chronic liver disease and in the development of HCC. The HCC risk induced by several types of carcinogens has been found to be profoundly reduced in gut-sterilized mice[54-56]. In a study exploring the differences between the gut microbiota of patients with nonalcoholic fatty liver disease (NAFLD)-related cirrhosis with and without HCC and in healthy controls, gut microbiota profile and systemic inflammation were significantly correlated and can occur together in the process of hepatocarcinogenesis[57]. It remains unclear whether chronic inflammation driven by the translocation of MAMPs from a leaky gut is the dominant contributor to HCC or whether the carcinogenic effect is limited to specific cases such as NAFLD, as does whether dysbiosis could serve as a causal link bridging CKD to HCC[58]. Further studies targeting dysbiosis may elucidate the association between CKD and HCC.

HCC and associated comorbidities causing renal dysfunction

A study found a significant prevalence of CKD in patients with cancer, particularly a higher rate of hematologic malignancy and liver cancer[59]. Hepatorenal syndrome (HRS), either with or without cirrhosis, is a major cause of CKD in patients with HCC. Direct invasion of the renal parenchyma by tumor cells is a rare cause but has been reported in the literature.

HRS: HRS is a unique type of kidney failure that usually occurs in advanced cirrhosis. HRS is characterized by functional impairment of the kidneys caused by vasoconstriction of the renal arteries in the absence of tubular dysfunction, proteinuria, or other histologic changes in the kidneys[54]. The exact mechanism of HRS is not completely understood, but its hallmark is severe vasodilation of the splanchnic arteries owing to portal hypertension, which compromises the effective arterial blood volume and arterial pressure[60]. HRS has two subtypes, which differ in terms of disease course and the presence of detectable precipitating factors[61]. Type 1 HRS is characterized by the rapid progression of renal failure, with the serum creatinine value increasing to greater than 2.5 mg/dL within 2 wk. It is often triggered by a precipitating event, such as bacterial infection, hypotension, or multiple organ failure. By definition, the renal dysfunction caused by type 1 HRS often falls into the category of acute kidney injury (AKI) or an acute deterioration of CKD termed acute-on-chronic kidney injury. By contrast, type 2 HRS is associated with gradual or insidious renal failure with a moderate rise in serum creatinine to 1.5-2.5 mg/dL. One of the major clinical manifestations of type 2 HRS is refractory ascites, for which a specific trigger is often lacking. With a median survival of 6 mo, Type 2 HRS has a superior prognosis compared with type 1 HRS, which has a median survival of less than 2 wk. The relatively moderate disease course is more consonant with the present article's focus on CKD.

Advanced cirrhosis, a critical precursor lesion of liver cancer, can cause portal hypertension, which may subsequently lead to HRS and result in kidney function deterioration[62]. In a 49-year-old man with HCC, ascites, and measured portal hypertension but no cirrhosis of the liver, the hypertension was secondary to microscopic invasion of the central and small portal veins[63]. Therefore, isolated liver cancer with high tumor burden has also been found to cause portal hypertension and HRS regardless of the presence of comorbid cirrhosis.

Direct tumor metastasis to the kidney: Renal metastasis of HCC is exceedingly rare. A literature search yielded only a few cases of renal metastasis from HCC[64]. Most renal metastases are small, bilateral, and multifocal; however, large and solitary metastatic tumors do occur. These tumors may cause difficulty in diagnosis because they often have no specific radiologic findings to distinguish them from primary renal neoplasms [65]. In some cases, metastatic tumors do not necessarily result in declined renal function. Nevertheless, in one case of HCC metastasis to the kidney mimicking renal cell carcinoma, a prolonged elevated serum creatinine level of 2.24 mg/dL was observed[66]. Therefore, direct invasion of the renal parenchyma by metastatic tumor cells still constitutes a differential diagnosis of renal dysfunction that should not be ignored in patients with HCC.

Paraneoplastic syndrome: Paraneoplastic syndromes arise from the tumor secretion of hormones, peptides, or cytokines or from immune cross-reactivity between malignant and normal tissues. These disorders may affect diverse organ systems, most notably the endocrine, nervous, dermatological, rheumatological, and hematological systems [67,68]. HCC may present with a wide range of paraneoplastic phenomena, which may precede local manifestations of the tumor, including hypercholesterolemia, erythrocytosis, hypoglycemia, and hypercalcemia. Hypercalcemia is a well-known paraneoplastic metabolic condition associated with numerous malignancies. In HCC, hypercalcemia accounts for 7.8% of paraneoplastic syndromes, and it mainly occurs as a terminal event[69]. Most malignancies associated with hypercalcemia have been verified to be caused by parathyroid hormone (PTH)-related peptide. The metastasis of malignancies to the bone can also cause osteolysis and lead to hypercalcemia. In rare cases, hypercalcemia may result from ectopic PTH production by tumors. Patients with hypercalcemia typically present with volume depletion, which might lead to a reduction in glomerular filtration rate (GFR) and calcium clearance[70]. Hypercalcemia may also provoke AKI or hypertension, or aggravate the tubular necrosis frequently found in cases of AKI[71]. Case reports on HCC-induced hypercalcemia have been published, but little information about renal function has been reported in these studies[72]. A case of combined HCC and neuroendocrine carcinoma with ectopic secretion of PTH was documented[73]; the authors observed impaired renal function (creatinine = 2.16 mg/dL), and continuous renal replacement therapy was applied to treat acute renal failure induced by hypercalcemia. However, the patient died during the study period. It is relatively certain that HCC may cause AKI through the paraneoplastic effect of hypercalcemia; nevertheless, more clinical observations and studies are warranted to determine whether HCC-related hypercalcemia causes sustained, even irreversible, renal dysfunction.

Risk factors shared by HCC and renal dysfunction

In the investigation of the relationship between CKD and HCC, some common risk factors have been found. The overlap of these risk factors leads to a high degree of comorbidities between HCC and renal dysfunction. These risk factors may cause the two diseases separately; however, little evidence exists for whether these factors serve as a causal link from HCC to CKD or vice versa. Hence, in this article we attempt to list these risk factors to provide clinicians and researchers with a useful summary. These risk factors can be further divided into several categories, including those of toxic, infectious, metabolic, and vascular origins (Table 1).

Environmental toxins: According to epidemiological and animal studies, several environmental toxins, including arsenic, cadmium, mycotoxins, and aristolochic acid (AA), are associated with both renal impairment and liver cancer[50].

In renal proximal tubules, arsenic or cadmium can cause the depletion of intracellular glutathione stores. This leads to the incremental production of free radicals and results in inflammation and apoptosis[74,75]. A high arsenic level in drinking water was discovered to be a cause for ESRD, independent of other documented risk factors[76]. Continual cadmium exposure can also progress to renal Fanconi syndrome and ultimately CKD[75]. By contrast, arsenic and cadmium carcinogenesis targets the liver[77]. Dimethylarsinic acid and trimethylarsine oxide, the organic metabolites of inorganic arsenic, have been found to cause oxidative DNA damage and enhance cell proliferation in rats[78,79]. In humans, arsenic exposure has also been potentially linked to HCC and other liver tumors or paraneoplastic lesions; for example, hepatomegaly, hepatoportal sclerosis, fibrosis, or cirrhosis often occurs after chronic arsenic exposure[80-82]. According to an *in vitro* experiment, cadmium is specifically internalized by Kupffer cells, which could lead to the release of various proinflammatory cytokines such as IL-6 and TNF-α[83,84]. Studies have also examined the potential effects of long-term cadmium exposure on the expression of cytochrome P450 (CYP) enzymes in the liver and its impact on the activation and clearance of therapeutic drugs, alcohol, and environmental substances. Under chronic cadmium exposure, DNA adducts associated with CYP-mediated metabolism are produced; they accumulate in liver cells and result in mutations, altered gene expression, and eventually carcinogenesis[85-87]. In epidemiological studies, elevated blood and urine cadmium levels have been found to play a role in HCC, although a direct effect has not been confirmed[77,88].

Aflatoxins (AFs) are highly toxic secondary metabolites that are synthesized by *Aspergillus flavus* and *Aspergillus parasiticu*[89]. Afs are the most toxic of all mycotoxins, causing considerable health problems and economic loss through the contamination of food and animal feed. Cereal crops, oil crops, and dairy products are frequently contaminated. Afs can be divided into AFB1 and AFB2, which emit blue fluorescence, and AFG1 and AFG2, which emit green fluorescence under chromatographic and fluorescence analysis[89]. Similar to cadmium, aflatoxin is metabolized by CYP enzymes into aflatoxin-8,9-exo-epoxide. The exo-epoxide can form derivatives with DNA, RNA, and proteins, including the p53 tumor suppressor gene. Moreover, the exo-epoxide can bind DNA to form the predominant promutagenic 8,9-dihydro-8-(N7 guanyl)-9-hydroxy AFB1 adduct (AFB1-N7-Gua), which may secondarily form the more mutagenic AFB1-formamidopyrimidine. These derivatives generate a risk of malignancy over time[89,90]. A review of the epidemiological evidence also indicated that AF is a critical contributor to the high incidence rates of HCC in Asia and Sub-Saharan Africa[91]. *In vitro* and *in vivo* studies have revealed that AFB1 and AFM1 cause kidney toxicity through oxidative stress by altering the expression of proline dehydrogenase and L-proline levels, leading to downstream apoptosis[89]. More population-based research is warranted to verify whether Afs are associated with renal dysfunction in humans. However, theoretically aflatoxin is likely to be a common risk factor shared by CKD and HCC.

Another factor worthy of discussion is AA. AA is traditionally known as the main culprit of Chinese herb nephropathy, a type of rapidly progressive renal failure characterized by severe anemia, glycosuria, leukocyturia, mild hypertension, and asymmetric kidneys[92]. Apart from being responsible for renal toxicity, AA has also been implicated in the genesis of urothelial carcinoma. AA-derived DNA adducts and TP53 mutations have been found in ureteric tissues, indicating the carcinogenic potential of AA on the urothelium[93,94]. AA can also result in significant DNA adduct formation and mutation in the liver, albeit at a lower level than in the kidneys [95]. Several epidemiological studies have implicated AA in the development of HCC in Asia, but more data are required to evaluate the impact of AA exposure on HCC occurrence worldwide[96,97].

Viral hepatitis: Chronic HBV and HCV infections are known to be dominant risk factors for HCC. HBV is the most frequent underlying cause of HCC. Case-control studies have demonstrated that chronic HBV carriers have a five- to fifteen-fold increased risk of HCC compared with the general population[98]. Approximately 70% to 90% of HBV-related HCCs develop in patients with cirrhosis, but HBV can also cause HCC in the absence of cirrhosis[99]. Generally, two processes are involved in the hepatocarcinogenesis of HBV infection. Direct mechanisms of hepatocyte transformation include a role for HBV DNA integration, virus mutations, transcriptional activation of growth regulatory genes by HBV-encoded proteins as well as effects on apoptosis, cellular signaling, and DNA repair. The progression of chronic hepatic disease and its associated inflammation, regenerative hyperplasia, and transcriptional deregulation to neoplasia contribute to the indirect pathogenesis of HCC[100,101]. By contrast, the mechanisms underlying HCV-associated carcinogenesis are mainly indirect effects of virus-deregulating host cellular processes, including virus-induced inflammation, oxidative stress, and host immune responses; the resulting genomic instability and mitochondrial damage; and the accompanying increased hepatocyte proliferation and steatosis[102].

In addition, HBV and HCV infection are also established risk factors for CKD. According to epidemiological studies, hepatitis B surface antigen positivity in serum is associated with higher risks of CKD and proteinuria[103]. HBV-related nephropathies include membranous glomerulonephritis, polyarteritis nodosa, and membranoproliferative glomerulonephritis (MPGN)[104]. Moreover, a clinical study reported that HBV causes apoptosis in renal tubular Fas upregulation[105]. HCV has been associated with the development of MPGN and cryoglobulinemia, and it has also been found to increase the risk of CKD[106,107]. Taken together, the aforementioned studies have indicated that both HBV and HCV can be considered critical shared factors in the high comorbidity of HCC and CKD. However, more research is required to verify whether these two infections are causally linked with HCC and CKD.

Metabolic diseases: Abundant epidemiological evidence suggests a correlation between noninsulin-dependent diabetes mellitus (NIDDM, or type 2 diabetes mellitus) and cancers, including HCC[108-110]. Several mechanisms likely explain such an association. Insulin or its precursors may stimulate mitogenesis or carcinogenesis in hepatocytes[111]. Augmented inflammation as measured by TNF-α and IL-6 levels has been found in diabetes[112,113]. Diabetes may also increase the risk of HCC through the development of NASH. Up to 40% of patients with type 1 or type 2 diabetes develop diabetic nephropathy, which is the leading cause of CKD in patients starting renal replacement therapy in developed countries[114]. Consequently, NIDDM is a non-negligible factor contributing to the high comorbidity of HCC and CKD. Likewise, fatty liver disease is a common risk factor for both HCC and CKD. The prevalence of NAFLD is 10%–30% in adults and tends to be higher in developed countries because of the prevalence of obesity and metabolic syndrome[115,116]. NASH belongs to the spectrum of NAFLD and is characterized by hepatic inflammation. In a study conducted to clarify the etiology of non-B, non-C HCC, a total 1374 patients with HCC were enrolled from 1995 to 2009. NASH was noted to be a critical risk factor for HCC, and cirrhosis was detected in 65% of NASH-HCC cases[117]. Studies have defined various factors involved in the necroinflammatory response of NASH, including cytokines, hormones, and neurotransmitters[118]. Rodent animal studies have demonstrated that NASH induced by a high-fat diet is associated with elevated TNF-α and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) with hepatocyte proliferation[113,119]. Hypoadiponectinemia possibly participates in NASH-HCC carcinogenesis, as verified by a adiponectin knock-out mice model[120]. Intriguingly, NAFLD was also found to be a driver of CKD[121]. The presence and severity of NAFLD were noted to be strongly and positively correlated with the prevalence and incidence of CKD, independent of obesity, hypertension, NIDDM, or other common risk factors[122]. Targher *et al*[123] found that higher levels of patatin-like phospholipase domain–containing protein 3 GG genotype are independently associated with a lower estimated GFR (eGFR) and increased 24-h proteinuria in patients with NAFLD. This single nucleotide polymorphism may be useful for identifying those patients with NAFLD who are also prone to developing CKD[123].

Vasoactive factors − activation of the renin–angiotensin system: The systemic renin–angiotensin system (RAS) regulates blood pressure and maintains normal kidney function. In addition to the traditionally known circulating RAS, scientists have uncovered the existence of a local angiotensin-generating system in several tissues, including the heart, liver, and kidney. The tissue RAS can act locally as a paracrine or

autocrine factor to meet the needs of individual tissues, independently of or in cooperation with the circulating counterpart[124]. The crucial role of the RAS in the pathogenesis of CKD has been well documented since the 1980s in experimental and clinical studies[125]. An activated RAS aggravates both systemic and glomerular capillary hypertension, causing hemodynamic injury to the vascular endothelium and glomerulus. Angiotensin II and the downstream product, aldosterone, also exert direct proinflammatory and profibrotic actions, which may promote kidney damage[126,127]. Angiotensin-converting-enzyme inhibition has exhibited considerable therapeutic efficacy in the control of systemic hypertension and the prevention of progressive kidney injury[128,129].

An increasing body of evidence has suggested that the RAS also contributes to liver fibrosis and hepatocarcinogenesis, although probably less so than it does for the kidneys[130]. The main source of the RAS comes from hepatocytes and Kupffer cells, but it has also been found in the bile duct epithelium of the liver[54]. In the liver, angiotensin II regulates cell growth, inflammation, and fibrosis. The expression of angiotensin receptors was found to increase activated hepatic satellite cells (HSCs) following injury. By acting through angiotensin receptors, angiotensin II can be mitogenic for human-activated HSCs, elicit a marked dose-dependent increase in intracellular calcium levels, and induce cell contraction. Angiotensin II also stimulates DNA synthesis and cell proliferation[131]. Angiotensin receptor blockers block the profibrotic and proinflammatory effects of angiotensin II on HSCs, including the expressions of inflammatory cytokines and growth factors (such as TGF-β1, IL-1β, NF-κB, and connective tissue growth factor) and the production of the extracellular matrix [132,133]. RAS might participate in the development of HCC because of the aforementioned proliferative and profibrotic effects. Moreover, angiotensin II was found to enhance vascular endothelial growth factor (VEGF), a potent angiogenic factor that plays an essential role in tumor growth and metastasis[134]. All these findings suggest that the RAS becomes involved in not only kidney injury but also HCC development.

EFFECT OF RENAL DYSFUNCTION ON THE OVERALL PROGNOSIS OF PATIENTS WITH HCC

Few studies have investigated the impact of comorbid renal dysfunction on the prognosis of patients with HCC. However, several studies have examined the influences of CKD or ESRD on specific treatment outcomes and prognosis, which are summarized in the next section. Before we discuss each of these topics in detail, we first provide a concise review of the literature on how renal dysfunction affects the prognosis of patients with HCC.

CKD increases the risk of death in cancer patients. A retrospective study investigating the association between CKD and mortality in cancer patients found an inverse relationship between eGFR and adjusted hazard ratios (HRs)[59]. A single-center study that recruited 440 patients with both CKD and HCC reported that survival from stage 4 and stage 5 CKD was inferior to that of stages 1 and 2. In a prospective population-based analysis, CKD was related to increased cancer-related mortality in liver, kidney, and urinary tract malignancies, with adjusted HRs of 1.74, 3.3, and 7.3, respectively[50]. However, in that study, the percentage of cancer-related mortality decreased, whereas the percentage of cardiovascular mortality markedly increased in patients in more advanced CKD stages. Taken together, we infer from these findings that CKD negatively affects both overall and cancer-related mortality in liver cancer, but some heterogeneity is possible in the etiology of mortality among different stages.

In terms of the prognosis of HCC patients with ESRD on long-term dialysis, studies have reported inconsistent results. In a single-centered observational study comparing the mortality rates of 1298 patients with HCC who were (n = 172) or were not (n = 1126) on long-term hemodialysis, those on hemodialysis had a 2.036-fold greater chance of death than did patients not on hemodialysis. However, cancer-related mortality was not reported and that study was limited by its retrospective nature and short follow-up duration[135]. In another single-center study including 2500 patients with HCC, with only a minority group (1.2%) having ESRD on maintenance dialysis, no significant overall survival difference between dialysis and nondialysis patients was found, although those receiving dialysis had a significantly higher serum bilirubin level, lower serum sodium level, more ascites, and worse performance status[136]. Because 63% of patients undergoing dialysis in that study had undergone nonpalliative management [resection, local ablation, or transarterial chemoembolization

(TACE)] for HCC, the authors attributed the unexpectedly good outcomes of the dialysis group to early and aggressive treatment. The authors further concluded that dialysis *per se* does not predict poor outcomes in patients with HCC and should not be considered a contraindication for active anticancer treatment. In summary, dialysis should not hamper the indicated group from receiving anticancer therapy according to currently available data, and whether dialysis affects the prognosis of HCC remains to be determined.

CHALLENGES OF TREATING HCC IN PATIENTS WITH RENAL DYSFUNCTION

Most patients with HCC have concomitant liver diseases such as chronic hepatitis or cirrhosis. Therefore, the benefits of treating the tumor must be weighed against the potential damage to liver function. This complexity in the management of HCC calls for a multidisciplinary approach, including expertise in hepatology, hepatobiliary surgery, pathology, oncology, radiology, and specialized nursing[137]. The BCLC algorithm classifies patients into one of five stages, taking not only the tumor burden but also the extent of liver dysfunction and the patients' performance status into consideration[138]. The tumor burden is quantified according to the number and size of nodules, along with the presence or absence of macrovascular tumor invasion or extrahepatic spread. The traditional Child–Turcotte–Pugh (CTP) score provides a subjective assessment of liver function but does not adequately capture the hepatic functional reserve. Alternatives include the Model for End-Stage Liver Disease (MELD) score and the albumin–bilirubin grade[139]. The algorithm then provides treatment recommendations for each stage. Ever since its release in 1999, the BCLC algorithm has been a widely used scoring strategy for HCC. In the very early (0) and early stage (A), patients with a solitary lesion or with up to three nodules less than 3 cm in diameter (without macrovascular invasion or extrahepatic spread) and with preserved liver function are suitable for radical therapies—namely resection, transplantation, or percutaneous treatment. Patients in the intermediate stage (B) do not exhibit symptoms but have large, multifocal tumors without vascular invasion or any spread beyond the liver. If liver function is preserved, these patients could be candidates for TACE. Patients at the advanced stage (C) have symptomatic tumors [grades 1 and 2 according to the Eastern Cooperative Oncology Group (ECOG) Performance Status] or an invasive tumoral pattern of vascular invasion/extrahepatic spread. This group of patients may benefit from systemic medical treatment, which can be categorized into targeted therapy and immunotherapy depending on which of the various pharmacological mechanisms are at work. Finally, patients with terminal disease (D) have poor liver function or marked cancer-related symptoms (ECOG Performance Status > 2). These patients have an extremely poor prognosis and require palliative care[140].

As mentioned in the previous section, CKD was reported to be an independent risk factor for the survival of cancer patients[59]. Treating HCC is difficult in patients with CKD because renal impairment may limit therapeutic options when effective therapy is sought[137]. Currently, perhaps because of the paucity of data regarding HCC outcomes in patients with renal dysfunction, no international treatment consensus exists for this specific population. In the following subsections, we use the BCLC algorithm as a template to discuss special concerns when treating HCC patients with different stages of renal dysfunction compared with the general population. Through reviewing the available literature, we hope to provide the necessary information for developing a modified BCLC for patients with CKD or ESRD (Table 1).

Liver resection

Liver resection is the treatment of choice in noncirrhotic patients and one of the main curative options for early HCC in selected patients with cirrhosis[141-144]. In the last decades, improved surgical techniques and perioperative management as well as improved patient selection have enabled the indications for liver resection to be expanded[145-148]. In a nationwide study using the National Surgical Quality Improvement Program database to investigate the impact of CKD and ESRD on outcomes following major abdominal surgery, 24572 patients were included, of whom only 149 (0.6%) were on hemodialysis preoperatively. In the dialysis group, 30-d postoperative mortality and the overall complication rate (pneumonia and sepsis particularly) were significantly higher than those in the nondialysis group. Furthermore, any degree of preoperative renal impairment, even mild or asympto-

matic disease, was associated with clinically significant increases in 30-d postoperative mortality and morbidity following major abdominal surgery[149]. Therefore, the safety and outcomes of liver resection in HCC patients with abnormal renal function deserve a detailed investigation.

Liver resection for HCC in patients with CKD: Few studies have reported on the efficacy and safety of hepatectomy for HCC patients with renal dysfunction. Toshima *et al*[150] retrospectively reviewed the clinical features of 722 patients with HCC undergoing curative hepatectomy between 1986 and 2009. Seventeen patients (2.4%) with preoperative serum creatinine levels > 2.0 mg/dL were defined as the renal dysfunction group. Clinicopathological characteristics and postoperative outcomes were compared between the renal dysfunction group (n = 17) and the nonrenal dysfunction group (n = 705). Overall survival (P = 0.177) and disease-free survival (P = 0.942) after hepatectomy did not differ significantly. The incidence rates of massive ascites (35.3% *vs* 14.3%; P = 0.034) and pleural effusion (52.9% *vs* 17.6%; P = 0.001), defined as massive effusion (ME), were significantly higher in the renal dysfunction group than in the nonrenal dysfunction group. Hypoalbuminemia (≤ 2.8 g/dL; P = 0.031), heavy blood loss (≥ 1000 mL; P = 0.012), and intraoperative blood transfusion (P = 0.007) were risk factors for ME. The authors concluded that preoperative improvement of anemia and reduction of blood loss by meticulous surgical techniques may prevent major complications in patients with renal dysfunction who require hepatectomy for HCC. In another study, data from 735 patients undergoing primary liver resection for HCC between 2002 and 2014 were analyzed[151]. Short- and long-term outcomes were compared between a renal dysfunction group, defined by a preoperative eGFR of < 45 mL/min/1.73 m^2, and a nonrenal dysfunction group. The incidence rates of postoperative pleural effusion (24% *vs* 11%; P = 0.007) and major complications (31% *vs* 15%; P = 0.003) were significantly higher in the 62 patients with renal dysfunction compared with the nonrenal dysfunction group. In patients with renal dysfunction with CTP score A, the 90-d mortality rate (1.9%) and median survival time (6.11 years) were comparable to those of patients without renal dysfunction. By contrast, patients with renal dysfunction with CTP score B had a very high 90-d mortality rate (22.2%), and a significantly shorter median survival time compared with patients without renal dysfunction (1.19 *vs* 4.84 years; P = 0.001). The authors concluded that liver resection is safe for CTP-A patients with renal dysfunction, who have comparable oncological outcomes to patients without renal dysfunction; however, liver resection for CTP-B patients with renal dysfunction should be subject to stricter consideration. These findings jointly indicate that CKD status may not necessarily affect overall survival but may lead to more surgical complications. The safety and efficacy of hepatectomy for HCC in patients with CKD could be acceptable if the appropriate patient group is carefully selected, along with judicious pre- and postoperative care.

Liver resection for HCC in patients with ESRD on dialysis: Compared with studies on the CKD population, studies on HCC patients with ESRD on dialysis undergoing hepatic resection are more abundant, probably because these patients' characteristics are well defined and more effectively targeted. To clarify the role of liver resection in treating HCC in patients with ESRD, Cheng *et al*[152] conducted a retrospective study to compare the clinicopathological characteristics and operative results of 12 patients with ESRD receiving resection for HCC with those of the other 456 patients without ESRD[152]. The 5-year disease-free survival rates for ESRD and non-ESRD groups were 35.0% and 34.2% (P = 0.31), whereas the 5-year overall survival rates were 67.8% and 53.3% (P = 0.54), respectively. The author commented that liver resection for HCC is justified in select patients with ESRD. In another retrospective study comparing the clinical features of 26 patients with ESRD and HCC with 1198 HCC patients without ESRD undergoing liver resection[153], elevated BUN and creatinine were the only two main independent factors differentiating patients with ESRD and HCC from their counterparts with HCC, and overall and disease-free survival rates were similar between the two groups. Lee *et al*[136] conducted a retrospective matched-control trial to compare long-term survival between patients with HCC (n = 2472) who were undergoing (n = 30) *vs* not undergoing dialysis[136]. The patients undergoing dialysis had dual HBV and HCV infection, lower serum α-fetoprotein level (AFP), worse performance status, and higher MELD scores than did the matched controls and patients not undergoing dialysis. No significant difference existed in long-term survival when patients undergoing dialysis were compared with patients who were not or with the matched controls (P = 0.684 and 0.373, respectively). Yeh *et al*[154] used Taiwan's National Health Institute Research Database to compare the disease-free

survival, overall survival, and perioperative complications between 596 nonuremic controls and 149 patients with uremia and HCC who were also undergoing liver resection. The survival outcomes were comparable between the uremia–HCC cohort and controls, regardless of the extent of hepatic resection. However, the aforementioned had a higher risk of postoperative infections requiring invasive interventions as well as an increased risk of life-threatening heart-associated complications relative to the controls. In summary, ESRD on dialysis does not seem to exert a particular influence on the survival outcomes of patients receiving liver resection for HCC. With careful operative techniques and perioperative care, comparable overall and disease-free survival can be achieved in select patients with ESRD and HCC undergoing liver resection. ESRD on dialysis is not expected to be an obstacle to hepatectomy in the indicated patient group.

Transplantation

Liver transplantation (LT) is considered the gold standard surgical therapy for early-stage HCC co-occurring with cirrhosis or chronic liver disease. The Milan criteria function as the most reliable border for transplantation feasibility both in Western and Asian HCC guidelines[155]. The expected 5-year survival rates of LT for HCC that meets the conventional Milan criteria (single tumor ≤ 5 cm or multiple tumors ≤ 3 nodules ≤ 3 cm in size, without vascular invasion) are 65%-80%, and patients meeting the Milan criteria have a significant survival advantage over patients who do not. LT is recommended as the first-line option for HCC within the Milan criteria but is unsuitable for resection. However, given the distinguished clinicopathological features of patients with renal dysfunction, whether the survival advantage of LT can be extended to this specific population is a more complicated matter. Can patients with renal dysfunction receive LT similar to the general population? How should one assess the feasibility of simultaneous liver kidney transplantation (SLKT)? The following paragraphs address these questions.

LT carries the risk of complications, which occur both immediately after transplantation and in the long term[156]. The main complications in the immediate postoperative period are related to graft dysfunction and rejection and to the surgical technique, infections, and dysfunction involved in the pulmonary, renal, or neurological systems. In the long term, complications are typically a consequence of prolonged immunosuppressive therapy, and they include diabetes mellitus, systemic arterial hypertension, de novo neoplasia, and organ toxicities[157]. AKI is a main complication of LT, especially in the early postoperative period. The reported incidence of AKI after transplantation varies widely because of the different diagnostic criteria used, ranging from 19.26% to 94%[158-161]. Hemodynamic changes during surgery, blood loss, and other stress may cause prerenal AKI or even acute tubular necrosis immediately after surgery[162,163]. Patients who developed AKI tended to have a markedly higher mortality rates[164,165]. It is unclear whether AKI after LT is the primary driver of poorer mortality outcomes or whether this is merely a correlation[166]. CKD is also a common complication after LT with an incidence ranging between 20% and 80%[167,168]. Numerous observations have implicated calcineurin-inhibitor (CNI) as a major risk factor of CKD in recipients of a transplant [169-171], and some studies have advocated the use of tacrolimus or mycophenolate mofetil instead of cyclosporin to reduce the incidence of chronic renal dysfunction after transplantation[170,172]. However, other studies have been unable to show that CNI fully explained post-transplant renal abnormalities[171,173]. Therefore, the hypothesis that CNI use is a major cause of renal dysfunction after LT remains unverified, and CNI's effect may be overestimated.

Patients with renal dysfunction have been reported to experience poor surgical outcomes following LT. An early study using the National Institute of Diabetes and Digestive and Kidney Diseases LT Database investigated the effect of renal insufficiency in patients with fulminant hepatic failure or chronic liver disease (cirrhosis); that study found that renal insufficiency in fulminant hepatic failure and renal insufficiency requiring dialysis or SLKT in cirrhosis predicts lower patient and graft survival rates after a transplant[174]. In another study reviewing the postoperative courses of 115 liver transplant recipients for liver cirrhosis, the population was divided into two groups based on the threshold of preoperative serum creatinine < 1.0 mg/dL[175]. Patients with preoperative serum creatinine > 1.0 mg/dL had significantly longer intensive care unit stays, higher rates of acute renal failure requiring dialysis, and a greatly increased mortality rate. In a study comparing the LT outcomes of patients with low and high MELD scores, renal function was the most crucial variable associated with morbidity and length of hospital stay[176]. The data not only called for

special attention during the perioperative period of renal dysfunction but also cast doubt on whether patients with renal dysfunction are ideal candidates for LT.

Several early studies have found that SLKT could be feasible in patients who have both advanced hepatic and renal dysfunction. In a study compared 16 patients with SLKT and 32 patients with LT matched by age, sex, date, and indication for transplantation; that study reported that both groups had similar levels of reoperation due to bleeding, bacterial infections, liver rejection, arterial hypertension, and median creatinine levels at the 1st and 3rd years[177]. However, early post-transplant dialysis was higher in SLKT than in LT. Survival rates at the 1st, 3rd, 5th, and 7th years were similar in both groups (87.5%, 74%, 74%, and 66% *vs* 81%, 75%, 75%, and 75% in LT and LKT, respectively). That author inferred that SLKT is an effective therapeutic option in patients with end-stage liver and kidney disease, with most early and late complications and long-term survival being similar to those observed in LT. In one study evaluating the success of SLKT, 20 patients (aged 14-64 years) received a total of 21 LT and 31 kidney transplantation procedures[178]. SLKT was performed in 14 patients, of whom five required further replacement of one or the other of the grafted organs. That study revealed that patients with liver cirrhosis had a very poor prognosis due to their poor overall clinical state at the time of terminal renal failure, whereas patients without liver cirrhosis were more appropriate candidates for SLKT. The author concluded that in general, the indication for SLKT ought to be considered earlier in this case than in the case of transplantation involving only one organ. Notably, a study found that pretransplantation renal dysfunction and exposure to dialysis might affect SLKT treatment outcomes[179]. Adult recipients receiving LT (n = 2700) or SLKT (n = 1361) with moderate renal insufficiency between 2003 and 2013 were included, and the study cohort was stratified into four groups based on serum creatinine level (Scr < 2 mg/dL *vs* Scr ≥ 2 mg/dL) and on dialysis status at both listing and transplant. SLKT administration led to a greater decrease in post-transplant mortality compared with LT administration across all four groups, but only reached statistical significance (HR 0.77; 95%CI: 0.62–0.96) in recipients not exposed to dialysis and with Scr ≥ 2 mg/dL at transplant. The study indicated the possible advantage of SLKT in patients with both severe liver disease and renal abnormalities. Some studies have indicated that the liver immunologically protects the kidneys after combined liver–kidney transplantation[180,181]. Therefore, patients with end-stage hepatic and renal anomalies may indeed benefit from SLKT. However, this technique faces limitations in being administered widely among HCC patients with renal dysfunction. For example, significant heterogeneity exists in the criteria for SLKT when it comes to noncirrhotic or compensated liver diseases and when it comes to liver transplant candidates with a moderate-to-severe reduction in GFR. To promote discussion and unify the criteria for the indication of SLKT by liver transplant groups, the Spanish LT Society (*La Sociedad Española de Trasplante Hepático*) held the 6th Consensus Document Meeting on October 20, 2016, in which experts from the 24 authorized Spanish LT programs participated[182]. According to the consensus, SLKT is recommended in patients with liver transplant criteria plus one of the following: (1) CKD in chronic dialysis or eGFR > 30 mL/min; or (2) CKD with eGFR between 30 and 40 mL/min and some signs of poor renal prognosis — such as proteinuria > 1 g/d (> 3 mo) and/or diabetic nephropathy — and/or histological findings of poor prognosis in renal biopsy (more than 30% glomerulosclerosis or more than 30% interstitial fibrosis). SLKT is also recommended in patients who are candidates for LT with acute kidney disease requiring dialysis for 6 consecutive weeks, either continuously or intermittently.

Several more recent studies have specifically focused on the outcomes of patients receiving SLKT for HCC. A study included 2606 patients (mean age: 53 years) receiving SLKT for primary biliary cirrhosis (PBC, n = 76), primary sclerosing cholangitis (n = 81), HBV (n = 98), HCV (n = 945), alcoholic liver disease (n = 495), alcohol and HCV (n = 152), cryptogenic cirrhosis (n = 289), NASH (n = 221), or HCC (n = 249); that study reported that HCV, NASH, and HCC had worse outcomes for liver graft (72%, 66%, and 72% *vs* 82%; HR: 2.5-3.1), kidney graft (71, 65%, and 71% *vs* 80%; HR: 2.3-2.8), and patient survival (74, 69, and 69% *vs* 82%; HR: 2.4-2.7) compared with PBC[183]. In another retrospective analysis of SLKT from the United Network for Organ Sharing registry[184], the authors compared the outcomes of HCC with other transplant indications. HCC was not associated with post-transplant survival among all patients (HR: 1.15; 95%CI: 0.84-1.58) or the propensity score-matched cohort (HR: 0.97; 95%CI: 0.64-1.47). SLKT-HCC patients had similar rates of acute rejection (13.3% *vs* 10.5%, P = 0.36) and liver graft failure requiring retransplantation (3.2% *vs* 2.3%, P = 0.44). The author commented that liver transplant candidates with advanced renal dysfunction and HCC may be considered for SLKT[184]. SLKT seems to be a treatment of choice for HCC patients with advanced renal dysfunction. However, more studies

specifically targeting patients with HCC as the main indication for SLKT are warranted to support the safety and efficacy of this treatment.

Radiofrequency ablation

Since the early 1990s, radiofrequency ablation (RFA) has been introduced to clinical practices and has rapidly become the first-choice local treatment for small (≤ 3 cm) HCC lesions. Based on the BCLC staging system, RFA is applied for the treatment of patients having very early (Stage 0) and early stage (Stage A) HCC (Figure 2)[138]. For most appropriate patients selected, this treatment is safe and efficient. However, reports of complications are common. Livraghi *et al*[185] and Takaki *et al*[186] reported mortality rates between 0.1% and 0.3%[185,186]. The major complication rate was estimated at 2.2% to 2.8%. The causes of death were bowel perforation, peritonitis, tumor rupture, and liver failure due to biliary stricture. The most frequent major complications were hemorrhage and tumor seeding, followed by liver abscess, bowel perforation, hemothorax, and liver failure. Minor complications included acute skin burn, self-limiting intraperitoneal bleeding, subcapsular or intrahepatic hematoma, arterioportal shunt, biliary portal shunt with hemobilia, transient liver decompensation, and direct renal tissue damage. In less common cases, the procedure may cause renal dysfunction or related side effects. Thermal injury could lead to hemolysis and rhabdomyolysis[187-189], and the extensive breakdown and transcellular shift of potassium may lead to varying (and even life-threatening) degrees of hyperkalemia, either in patients with normal baseline renal function or CKD[190]. This clinical implication is anticipated in case of prolonged ablation, and laboratory monitoring during extensive or prolonged RFA procedures is recommended to detect hemolysis early. Laboratory tests including hematocrit, serum potassium, urine hemoglobin, and serum creatine phosphokinase level should be considered[188]. Hemolysis and rhabdomyolysis could also result in AKI[188,189,191,192]. Most patients experience moderately impacted renal function and a slight increase in serum creatinine without deterioration. However, the hemoglobin-mediated obstruction of renal tubules might cause more severe AKI, oliguria, and sometimes even death. One case report even documented progression to CKD[193].

Few original studies or systematic reviews have discussed whether pre-existing renal dysfunction before RFA is related to treatment outcomes, although much more evidence indicating treatment outcomes in patients with ESRD on dialysis receiving RFA for HCC have been emerging. To examine the efficacy and safety of RFA in treating HCC in patients with HD, a study enrolled 108 HD patients with naïve HCC at 15 institutions between 1988 and 2014[194]. Fifty-eight patients with appropriate indications treated with either hepatectomy (n = 23) or RFA (n = 35) were compared with respect to their clinical features, complications, and prognosis. The two treatments did not significantly differ in their overall survival and disease-free survival rates. The author concluded that RFA had a therapeutic efficacy in HD patients with naïve HCC that is comparable to liver resection. Another study included 14 carefully selected HD patients with HCC (five naïve, nine recurrent) who underwent a total of 19 RFA treatments, and revealed no major complications, suggesting that the safety and effectiveness of RFA were not compromised in this specific population[195]. RFA seems to be a promising option for small HCC in patients undergoing regular HD. By contrast, a study using the Japanese Diagnosis Procedure Combination database compared the treatment outcomes in matched-pair samples of 437 dialyzed and 1345 nondialyzed patients[196]. In-hospital mortality and hemorrhagic complications were significantly higher in dialyzed patients with ESRD than in nondialyzed patients. In patients on HD for ESRD, mortality was significantly lower for those aged ≤ 70 years than for those aged older than that (P = 0.02). Patient age may be a useful indicator when considering RFA for HCC in patients with ESRD on HD. Hyperkalemia was also reported in a patient with ESRD on regular HD after RFA for HCC[197]. Therefore, the indications for RFA in dialysis-dependent patients should be considered carefully.

TACE

Transarterial therapy is a standard treatment for unresectable HCC and patients unfit for surgical resection due to compromised hepatic reserve or nonliver general comorbidities[138,198], following which regular contrast-enhanced imaging for residual disease is recommended. The chemotherapeutic agents used in TACE cause tumor necrosis through the combined effects of targeted chemotherapy and arterial embolization[199]. However, the use of a water-soluble iodinated contrast medium in TACE may induce renal failure, especially in high-risk patients with liver cirrhosis-associated nephropathy[200]. AKI is a common complication found after TACE in

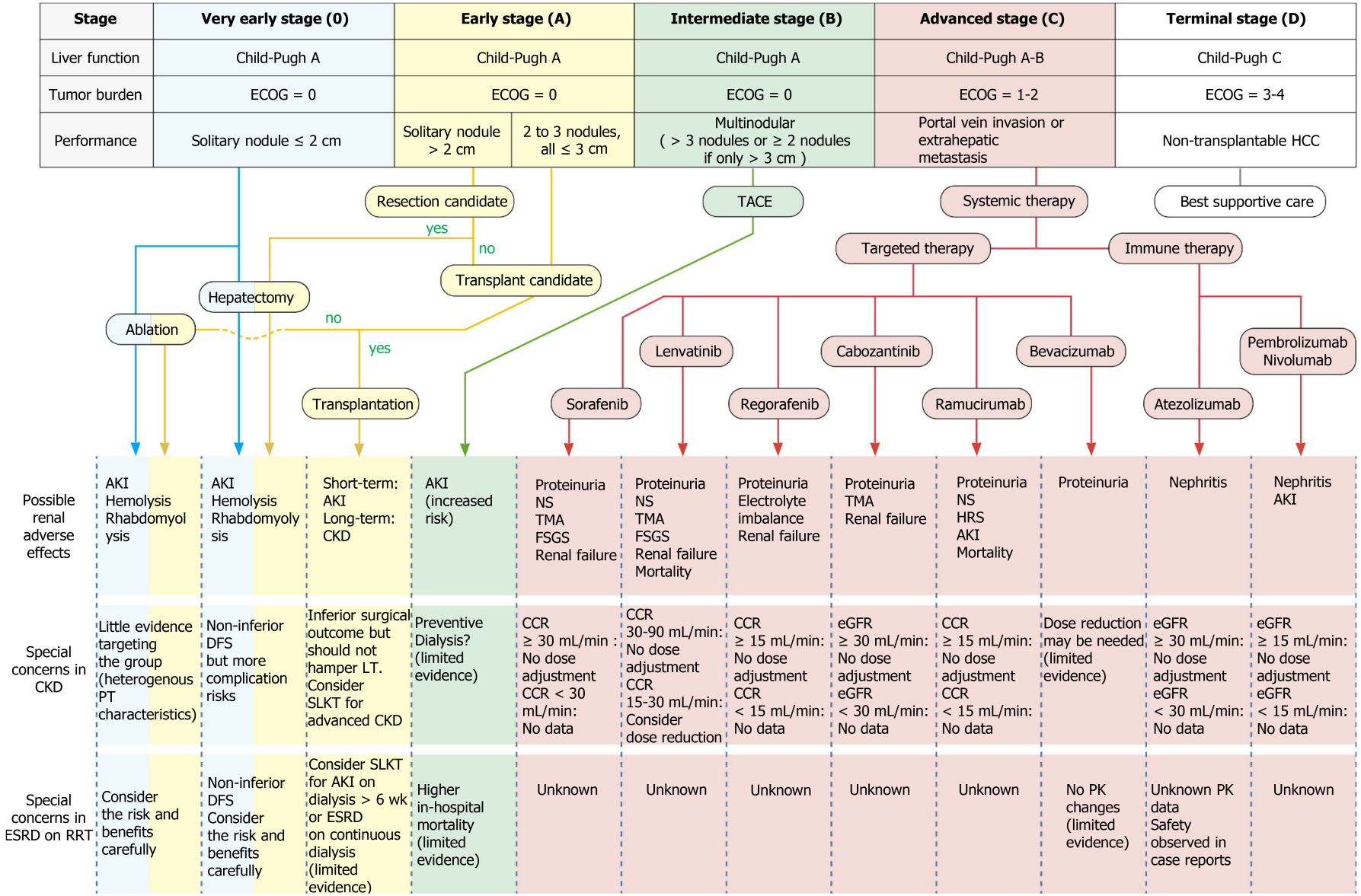


Figure 2 Current treatment algorithmsfor patients with hepatocellular carcinoma and chronic kidney disease. This figure is based on the Barcelona Clinic Liver Cancer algorithm, which classifies patients into five stages taking not

only the tumor burden but also the extent of liver dysfunction and the patients' performance status into consideration. Described in the table are the indications for each stage of hepatocellular carcinoma treatment, the treatment-related renal adverse effects, and special concerns for patients with pre-existing renal dysfunction in the applying aggressive treatments and the use of the systemic target and immunotherapy. The disease-free survival is compared to the general population without renal impairment. ECOG: The Eastern Cooperative Oncology Group performance status; HCC: Hepatocellular carcinoma; CKD: Chronic kidney disease; ESRD: End stage renal disease; RRT: Renal replacement therapy; AKI: Acute kidney injury; PT: Patient; LT: Liver transplantation; SLKT: Simultaneous liver kidney transplantation; DFS: Disease-free survival; NS: Nephrotic syndrome; TMA: Thrombotic microangiopathy; FSGS: Focal segmental glomerulosclerosis; CCR: Creatinine clearance (mL/min calculated *per* the Cockcroft-Gault formula); eGFR: Estimated glomerular filtration rate (mL/min/1.73 m^2); PK: Pharmacokinetics.

patients with HCC, and patients with post-TACE AKI have a higher risk of developing complications such as progression to CKD, ESRD, and death[200-204]. Preoperative CTP score, age, proteinuria, hemoglobin, serum total bilirubin, serum uric acid, aminotransferase level, post-TACE gastrointestinal bleeding, and previous post-TACE AKI history have been reported to be predictors of post-TACE AKI in HCC patients [201,205,206].

Given the nephrotoxicity inherent in the intervention, the application of TACE for HCC in patients with underlying renal dysfunction is challenging. According to a retrospective study that investigated the outcomes of TACE in patients with HCC and CKD, more post-therapy complications, including acute renal failure and sepsis, were found in the CKD group than in the non-CKD group[207]. Overall survival in the CKD group was significantly poor (10.9 ± 8.5 *vs* 23.5 ± 16.3 mo, $P < 0.01$). However, in another study conducted to clarify the benefits and risk of TACE in patients with HCC and CKD, 35 patients receiving TACE were enrolled and classified into a CKD group [including nondialysis CKD (NDCKD), $n = 10$ and ESRD, $n = 9$], and a non-CKD group ($n = 16$)[208]. The 2- and 5-year survival rates from initial diagnosis were comparable between the CKD and non-CKD groups. The 2- and 5-year survival rates were also similar in patients with NDCKD and those with ESRD. Of note is the strategy of "preventive HD" adopted in that study: All patients with CKD consulted a nephrologist, and HD was performed within 4 h after 20 of the 32 transarterial therapies in the 10 patients with NDCKD to prevent contrast-induced nephropathy in the CKD group. The authors concluded that TACE can be made feasible in patients with CKD by instituting periprocedural HD with survival rates that are similar to those of patients without CKD.

For patients already on regular hemodialysis for ESRD at the time of TACE, data are lacking because invasive treatment is rarely performed in this specific population. A Japanese pair-matched cohort using a nationwide database was recruited to evaluate the in-hospital mortality and complication rates following TACE in this population [209]. A total of 1551 dialyzed and 5585 nondialyzed patients with ESRD were enrolled. The complication rates did not differ between dialyzed and nondialyzed patients, but the in-hospital mortality rate was, at 2.2%, twice as high in dialyzed patients. Among the dialyzed patients, the mortality rate was not significantly associated with sex, age, or Charlson Comorbidity Index. The author concluded that indications for TACE in HD-dependent patients should be considered cautiously by weighing the benefits against the risks.

In summary, the available data regarding TACE in patients with pre-existing renal dysfunction are limited. More studies are warranted before we can definitely determine the safety and feasibility of TACE in patients with CKD or ESRD. Patients with advanced renal dysfunction may benefit from perioperative preventive HD, but further investigations are required to confirm the efficacy and safety of the measure. Because both the CKD and ESRD groups have reported worse prognoses after TACE compared with HCC patients without renal dysfunction, caution should be taken during the treatment planning process, and patients should be well-informed of the risks and complications involved.

Systemic therapy

If HCC is diagnosed at an early stage, a wide array of treatment options that increase overall survival and improve quality of life are available. However, because late diagnosis is common, 70% to 80% of advanced HCC cases will not benefit from tumor resection[3], and only one-third of patients are eligible for curative therapeutic approaches[210]. Current treatment options for patients with unresectable HCC include TACE and systemic medical treatments. Systemic treatments can generally be divided into two categories according to their mechanism of action: Targeted therapy [mainly tyrosine kinase inhibitor (TKI)] and immunotherapy. The following subsections concentrate on the mechanism of action, common adverse effects, and points of caution for people with renal dysfunction with respect to two groups of drugs.

Targeted therapy: The key signal transduction pathways participating in the pathogenesis of HCC include the Wnt-β catenin, EGFR-RAS-MAPK, and c-MET pathways as well as the insulin-like growth factor signaling, Akt/mTOR signaling, and VEGF and platelet-derived growth factor receptor signaling cascades[211]. TKIs are small molecules that inhibit the multiple receptor tyrosine kinases involved in tumor growth, angiogenesis, pathologic bone remodeling, drug resistance, and metastatic progression of cancer[204]. In 2007, a multi-kinase inhibitor (MKI) named sorafenib was approved as the first systemic agent for treating advanced unresectable HCC because a SHARP trial had suggested a survival benefit of approximately 3 mo [212,213]. Sorafenib is an oral MKI that blocks tyrosine kinase receptors (VEGFR-2/3, PDGFR-β, c-Kit, FLT-3, and RET) and other targets (c-Raf and B-Raf)[214]. In the kidneys, glomerular podocytes express VEGF and glomerular endothelial cells express VEGF receptors[215,216]. Podocyte-specific deletion of a single VEGF allele caused proteinuria and capillary endotheliosis in rodents, and disrupted glomerular VEGF signaling was strongly implicated in the pathogenesis of human preeclampsia[208]. Sorafenib's mechanism of action clearly indicates its ability to induce significant adverse effects on the kidneys, including proteinuria, nephrotic syndrome, and preeclampsia-like syndrome[217,218]. Cases of renal failure, thrombotic microangiopathy (TMA), and focal segmental glomerulosclerosis (FSGS) have also been documented[213,219]. In patients on sorafenib with pre-existing renal dysfunction, studies have found no trend in pharmacokinetic parameters for sorafenib or its metabolites among any renal function group[220,221]. Renal impairment appears to have no clinically relevant effect on the pharmacokinetics of sorafenib and its metabolites; therefore, no dose adjustment was indicated[221]. According to the Food and Drug Administration (FDA), the pharmacokinetics of sorafenib have not been thoroughly confirmed in patients on dialysis[222]. However, an Italian retrospective study investigating the safety and efficacy of sorafenib in patients with renal cell carcinoma and ESRD reported no unexpected major side effects, and the author concluded that sorafenib is not contraindicated in HD groups[223]. In a Japanese study, a 63-year-old man with ESRD on HD started sorafenib therapy (200 mg/d) 8 d after TACE[221]. The pharmacokinetic parameters of sorafenib and its active metabolite M-2 were within the reference levels of patients with normal renal function 8 and 9 d after the initiation of sorafenib. The authors concluded that sorafenib was well tolerated at an initial dose of 200 mg/d for a patient with HCC undergoing HD, thus indicating that renal failure is not necessarily a contraindication for sorafenib therapy.

After the success of sorafenib, various clinical trials were designed in the hope to outperform the efficacy of it. Nevertheless, not until in recent decade had some trials demonstrated the comparable efficacy with sorafenib or survival benefits after first-line treatment failure[224]. The notable novel agents include lenvatinib, regorafenib, cabozantinib, ramucirumab, and bevacizumab.

Lenvatinib was approved for first-line therapy in advanced HCC following the results of the REFLECT trial, a randomized phase III noninferiority trial by Kudo *et al* [225], which showed that lenvatinib was not inferior to sorafenib in overall survival in untreated advanced HCC[225]. Further multicenter findings have confirmed the efficacy of lenvatinib with or without previous TKI therapies[226,227]. Lenvatinib's nephrotoxic profile is similar to that sorafenib, including proteinuria, renal failure, TMA, and FSGS[225,228-231]. The enrollment criteria in the original REFLECT trial included adequate renal function, which was defined as creatinine clearance (CCR) > 30 mL/min as calculated using the Cockcroft–Gault formula[225]. In the FDA label, no dose adjustment is recommended for patients with mild (CCR 60-89 mL/min) or moderate (CCR 30-59 mL/min) renal impairment. Lenvatinib concentrations may increase in patients with differentiated thyroid cancer (DTC) or renal cell carcinoma (RCC) and severe (CCR 15-29 mL/min) renal impairment. It is recommended to reduce the dose for patients with DTC or RCC who also have severe renal impairment. However, there exists no recommended dose for lenvatinib in patients with HCC and severe renal impairment. Lenvatinib has not been studied in patients with ESRD[232].

Regorafenib was approved as the second-line therapy for advanced HCC following the results of the RESORCE trial. This randomized, double-blind, placebo-controlled phase III trial demonstrated the effectiveness of regorafenib in patients progressing after sorafenib treatment. The study confirmed the potential of second-line agents and ushered in the era of second-line therapy[233]. Further multicenter studies have verified the efficacy and safety indicated in the RESORCE trial[234,235]. The nephrotoxic effects include proteinuria and renal failure[236,237]. In regorafenib monotherapy for previously treated metastatic colorectal cancer (CORRECT), an international, multicenter, randomized, placebo-controlled, and phase III trial reported diarrhea in 34% of patients, with 7% experiencing grade 3 or 4 diarrhea, leading to fluid and electrolyte depletion. The sequelae of fluid and electrolyte depletion may result in dehydration, renal failure, and potential cardiovascular compromise[238]. According to a pharmacokinetic modeling and simulation study, the pharmacokinetics of regorafenib are unlikely to be impacted by any stage of renal impairment[239]. The FDA label suggests that no dose adjustment is recommended for patients with renal impairment. The pharmacokinetics of regorafenib have not been studied in patients on dialysis and there exists no recommended dose for this patient population[240].

Cabozantinib is another TKI that blocks the receptors involved in oncogenesis and angiogenesis, including VEGFR 1, 2, and 3; hepatocyte growth factor receptor (MET); AXL; and the angiopoietin receptors TIE-2, RET, c-Kit, and FLT-3 *in vitro* and *in vivo*. Cabozantinib was also indicated to be a second-line treatment in the progression of HCC with acquired resistance to sorafenib[241]. In the CELESTIAL trial, cabozantinib achieved significantly superior overall survival compared with the placebo group and was thus approved by the FDA[242]. The nephrotoxic profile of cabozantinib is similar to those of sorafenib and lenvatinib, including renal failure, proteinuria, and TMA[243-245]. However, in the CELESTIAL trial, grade 5 adverse events considered to be related to the drug were reported in six patients in the cabozantinib group (one event each of hepatic failure, bronchoesophageal fistula, portal-vein thrombosis, upper gastrointestinal hemorrhage, pulmonary embolism, and HRS)[242]. The enrollment criteria for the CELESTIAL trial included serum creatinine ≤ 1.5 times the upper normal limit or calculated CCR ≥ 40 mL/min using the Cockcroft–Gault formula occurring in conjunction with either urine protein/creatinine ratio ≤ 1 mg/mg or 24-h urine protein < 1 g[242]. Two clinical pharmacology studies were conducted to characterize the single-dose pharmacokinetics of cabozantinib in individuals with renal and hepatic impairment, respectively[243]. Although mild-to-moderate renal impairment (eGFR ≥ 30 mL/min/1.73 m^2) did not result in a clinically relevant difference in the pharmacokinetics of cabozantinib, the author concluded that cabozantinib should be used cautiously in individuals with mild or moderate renal impairment. According to the FDA label, no dose adjustment is recommended in patients with mild or moderate renal impairment[246]. No experience of cabozantinib in patients with severe renal impairment or requiring dialysis has been documented.

Ramucirumab is a fully human recombinant immunoglobulin G (IgG) 1 monoclonal antibody targeting the VEGF2 receptor. A randomized, multicenter, double-blind, placebo-controlled, and phase III trial (REACH) was conducted to examine the safety and efficacy of ramucirumab as a second-line agent for HCC[247]. In the REACH trial, although the second-line treatment with ramucirumab did not significantly improve survival over placebo in patients with advanced HCC, a subgroup analysis revealed better survival in patients with AFP ≥ 400 ng/mL[248,249]. This was later verified in the REACH-2 trial, which was the first positive phase III trial conducted in a biomarker-selected patient population with HCC[250]. Therefore, ramucirumab was

approved by the FDA as a second-line treatment for advanced HCC. The renal toxicity profile of ramucirumab includes proteinuria and nephrotic syndrome[251,252]. Renal failure and TMA have also been reported[250,253]. Notably, several predictors of ramucirumab-induced proteinuria have been identified, including systemic blood pressure, the number of cycles, and calcium channel blocker use[248,254]. Notably, in the REACH-2 trial, three deaths in the ramucirumab group were judged to be related to study treatment: one each from AKI, HRS, and renal failure[250]. The FDA label reports no clinically meaningful effect on the pharmacokinetics of ramucirumab in patients with renal impairment (CCR calculated using Cockcroft–Gault, 15-89 mL/min), and thus, no dose adjustment is suggested[255]. The pharmacokinetics of ramucirumab in patients with ESRD are unknown.

Bevacizumab is a humanized anti-VEGF monoclonal antibody that was previously approved by the FDA as a first-line treatment for metastatic colorectal cancer in combination with chemotherapy. In a global, open-label, and phase III trial conducted in 2020, patients with unresectable HCC who had not previously received systemic treatment were randomly assigned at a 2:1 ratio to receive either atezolizumab (discussed later in the text) plus bevacizumab or sorafenib until unacceptable toxic effects or a loss of clinical benefit occurred[256]. The primary end points were overall survival and progression-free survival in the intention-to-treat population, as assessed at an independent review facility according to the Response Evaluation Criteria in Solid Tumors, version 1.1. The study revealed that in patients with unresectable HCC, atezolizumab combined with bevacizumab resulted in superior overall and progression-free survival outcomes than sorafenib did, which led to the combined therapy of atezolimumab plus bevacizumab being approved as the first-line treatment for unresectable HCC by the FDA. Similar to other anti-VEGF or VEGFR blocking agents, the renal toxicity profile of bevacizumab encompasses renal failure[257,258], proteinuria, and nephrotic syndrome[259,260]. Microvascular diseases such as TMA or hemolytic uremic syndrome are not particularly uncommon[261-263]. In addition, sporadic cases of interstitial nephritis have been documented, as verified by renal biopsy findings, improvement after steroid treatment, and cessation of the offending agents[264,265]. A case of minimal change disease was also reported[266]. *Per* the manufacturer's instructions, no studies have investigated the pharmacokinetics of bevacizumab in patients with CKD because the kidneys are not major organs for bevacizumab metabolism or excretion[267]. Only one report has been published about the pharmacokinetics of bevacizumab in a dialysis-dependent patient with metastatic renal cancer, who received 5 mg/kg every 2 wk[268]. The drug was not dialyzable, and its pharmacokinetic parameters were similar to the reference values of patients with normal renal function. The author concluded that the drug can be administered any time before or after hemodialysis. The FDA label does not provide information on dose adjustment in patients with renal dysfunction[267].

Immunotherapy: Immunotherapy has been proven to be effective and safe in treating various solid tumors, prolonging overall survival, and offering a tolerable toxicity profile[269]. Immunotherapy negates tumor-expressed extracellular ligands that suppress intrinsic immune response and can be achieved through three main approaches[270]. One approach is to target the inhibitory proteins that prevent T cells from recognizing and eliminating cancer cells and allow regulatory cells to avoid autoimmune destruction by downregulating T-cell activation[269]. Examples of these molecules are cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), programmed cell death protein-1 (PD-1) in addition to its ligand PD-L1, and T cell immunoglobulin and mucin domain-containing protein 3 (TIM-3)[271,272]. Checkpoint inhibitors are antibodies that activate T-cell mediated antitumor responses by selectively blocking the checkpoint receptors PD-1, PD-L1, and CTLA-4[271]. Conversely, therapeutic cancer vaccines that use a tumor-associated antigen (TAA) originating either from whole-cell tumor lysates and recombinant tumor peptides or recombinant viruses encoding for TAAs bring new prospects in treating cancers. TAAs are transferred and presented by major histocompatibility complex class I molecules in atrial premature complexes to effectively induce the activation of cytotoxic T-lymphocytes[273,274]. Another strategy in immune-regulated antitumor response is that of adoptive cell transfer. Immune cells are extracted from patients' peripheral blood and undergo genetic engineering to express chimeric antigen receptors. These cell membrane proteins bind to specific cancer antigens and stimulate the immune destruction of tumor cells[275].

In HCC, two categories of immune checkpoint inhibitors have been thoroughly examined in clinical trials, namely PD-1/PD-L1 and CTLA-4. Other promising markers are being investigated in animal models, and new agents are being tested in

clinical trials[272]. Currently, the FDA has approved checkpoint inhibitors for advanced HCC, including atezolizumab, pembrolizumab, nivolumab, and ipilimumab. The following paragraphs discuss the mechanism of action, common adverse effects, and points of caution for people with renal dysfunction in the use of these agents. The anticipating checkpoint molecule TIM-3 blockade and the related clinical trials are mentioned as well.

Atezolizumab is an engineered IgG1 monoclonal antibody targeting PD-L1. Patients with unresectable HCC who had not previously received systemic treatment were randomly assigned at a 2:1 ratio to receive either atezolizumab plus bevacizumab or sorafenib until unacceptable toxic effects or a loss of clinical benefit occurred[256]. Atezolizumab combined with bevacizumab resulted in superior overall and progression-free survival outcomes than did sorafenib, which led to the FDA approving the combined therapy of atezolizumab plus bevacizumab as the first-line treatment for unresectable HCC (see the preceding paragraph on bevacizumab). In a study focusing on the use of atezolizumab in patients with renal insufficiency, the efficacy and safety of atezolizumab in these special subpopulations from an expanded access program was reported[276]. Objective responses occurred in 0/6 (0%), 4/19 (21%), 1/27 (3.7%), and 12/62 (19%) of evaluable patients with CCR < 30, 30-45, 45-60, and ≥ 60 mL/min, respectively, and stable disease course was observed in three patients with CCR < 30 mL/min. The author concluded that these findings verified the clinical benefit of atezolizumab in patients with compromised renal function. In one case report, a male patient with metastatic urothelial cell carcinoma and ESRD on dialysis was safely treated with atezolizumab[277]. The main kidney-related side effect caused by atezolizumab is acute tubulointerstitial nephritis, as reported in biopsy-proven cases[278,279]. Based on the FDA label, mild or moderate renal impairment (eGFR 30-89 mL/min/1.73 m^2) has no clinically significant effect on systemic exposure to atezolizumab; however, the effects of severe renal impairment (eGFR < 30 mL/min/1.73 m^2) or severe hepatic impairment on the pharmacokinetics of atezolizumab is unknown[280].

Pembrolizumab is an anti-PD-1 monoclonal antibody. The antitumor effects of pembrolizumab were examined in a phase II trial in patients who were previously treated with advanced HCC (KEYNOTE-224)[281]. Subsequently, a randomized, double-blind, and phase III study (KEYNOTE-240) was conducted to further verify the efficacy and safety of pembrolizumab in this population[282]. The study indicated a favorable risk-to-benefit ratio for pembrolizumab in this population, but the overall and progression-free survival did not reach statistical significance *per* the specified criteria. Based on the aforementioned trials, the FDA granted accelerated approval to pembrolizumab for patients with HCC who have been previously treated with sorafenib. Several adverse renal effects have been noted during the use of pembrolizumab, including acute tubular injury, acute interstitial nephritis, and minimal change disease; moreover, kidney biopsy was recommended for suspected pembrolizumab-related cases of AKI[283-285]. According to the FDA label, regarding the risk of immune-mediated nephritis, changes in renal function should be monitored during use. The FDA also advised withholding pembrolizumab and administering corticosteroids for grade 2 nephritis or higher, and they also advised permanently discontinuing the drug for severe (Grade 3) or life-threatening (Grade 4) nephritis. Renal impairment (eGFR ≥ 15 mL/min/1.73 m^2) has no clinically significant effect on the clearance of pembrolizumab. Insufficient information exists regarding whether clinically important differences exist in the clearance of pembrolizumab in patients with eGFR < 15 mL/min/1.73 m^2[286].

Nivolumab is another anti-PD-1 monoclonal antibody. An open-label, noncomparative, phase 1/2, and dose escalation and expansion trial (CheckMate-040) was conducted to assess the safety and efficacy of nivolumab in patients with advanced HCC with or without chronic viral hepatitis[287]. The FDA later granted accelerated approval to nivolumab for patients with HCC who have previously been treated with sorafenib. A randomized, multicenter phase III study (CheckMate-459) of nivolumab *vs* sorafenib in patients with advanced HCC is currently ongoing to examine the use of nivolumab as a first-line treatment[288]. The renal toxicity profile of nivolumab is similar to that of pembrolizumab, including AKI, acute tubular injury, and immune complex-mediated glomerulonephritis[284,289,290]. The FDA label contains special warnings, prescribes precaution for immune-mediated nephritis and renal dysfunction during use, and advises that patients should be monitored for changes in renal function. The drug should be withdrawn in cases of moderate or severe serum creatinine elevation and permanently discontinued in cases of life-threatening serum creatinine elevation. The effect of renal impairment on the clearance of nivolumab was evaluated through a population pharmacokinetics analysis in patients with mild

(eGFR: 60-89 mL/min/1.73 m^2), moderate (eGFR: 30-59 mL/min/1.73 m^2), or severe (eGFR: 15-29 mL/min/1.73 m^2) renal impairment. No clinically important differences in the clearance of nivolumab were found between patients with renal impairment and those with normal renal function. The FDA suggests no dose adjustment in patients with renal impairment[291].

Ipilimumab is a CTLA-4 immune checkpoint inhibitor. The anti-HCC effect of ipilimumab was demonstrated in the nivolumab plus ipilimumab cohort in CheckMate-040, a multicenter, open-label, and phase 1/2 study (described in the preceding paragraph on nivolumab)[292]. The FDA granted accelerated approval to the combination of nivolumab and ipilimumab for patients with HCC who have previously been treated with sorafenib. As with those for other immunotherapy agents, evidence of the adverse renal effects of ipilimumab has indicated their presence in the forms of acute interstitial nephritis and AKI, constituting a cause for alarm[293]. However, the onset of kidney injury as indicated by CTLA-4 antagonist-related renal injury occurs earlier (2–3 mo) than that indicated by PD-1 inhibitors (3–10 mo)[284,294]. Furthermore, a case of ipilimumab-induced lupus nephritis was also reported[295]. Notably, ipilimumab has also been associated with electrolyte disturbances. Ipilimumab-induced hyponatremia caused by pituitary hypophysitis has been documented in case reports[296,297]. The FDA label suggests that patients should be monitored for changes in renal function. Furthermore, the drug should be withdrawn in cases of moderate or severe serum creatinine elevation and permanently discontinued in cases of life-threatening serum creatinine elevation. The effect of renal impairment on the clearance of ipilimumab was evaluated in patients with mild (eGFR: 60-89 mL/min/1.73 m^2), moderate (eGFR: 30-59 mL/min/1.73 m^2), or severe (eGFR: 15-29 mL/min/1.73 m^2) renal impairment compared with patients with normal renal function (eGFR: ≥ 90 mL/min/1.73 m^2) in a population pharmacokinetics analysis. No clinically important differences in the clearance of ipilimumab were found between patients with renal impairment and patients with normal renal function. The FDA recommended no dose adjustment for patients with renal impairment[298].

In addition to antibodies against CTLA-4 and PD-1/PD-L1, checkpoint inhibitor targeting TIM-3 is another potential and promising candidate of immunotherapy for cancer treatment[272,299]. TIM-3, a type I surface glycoproteins encoded by the gene on chromosome 5q33.2, was first discovered in 2001 and identified as an immune checkpoint that specifically expressed on interferon-γ-secreting CD4(+) T helper 1 and CD8(+) T cytotoxic cells in both mice and humans[300,301]. TIM-3 acts as a negative regulator of T cell function by triggering cell death upon interaction with its ligand, galectin-9. TIM-3 overexpression has been implicated in the suppression of T-cell responses and T-cell dysfunction; a state referred to as T-cell exhaustion[302]. TIM-3 also has other ligands and is expressed on other cell types like dendritic cells[303], monocytes[304], and mast cells[305]. In chronic HBV infection, TIM-3 expression is elevated in T helper cells, cytotoxic T lymphocytes, dendritic cells, macrophages, and natural killer cells, accompanied by impaired function of these immunocytes[306]. The TIM-3/galectin-9 signaling pathway was found to mediate T-cell senescence in HBV-associated HCC[307]. In addition to the immunomodulation effect, the expression of TIM-3 on tumor cells has been found to regulate the function of tumor cells directly [308]. A mechanistic study showed that TIM-3 expressed by malignant hepatocytes served as a tumor cell-intrinsic receptor to promote tumor growth *via* triggering NF-κB/IL-6/STAT3 axis[309]. Therefore, TIM-3 is a drug target for treating both chronic viral infection and HCC.

Several clinical trials about the use of anti-TIM-3 monoclonal antibodies in different types of cancer have been registered on ClinicalTrials.gov. MBG453, an anti-TIM-3 monoclonal antibody, was tested for the safety and efficacy of a single agent or in combination with PDR001 (anti-PD-1 antibody) in adult patients with advanced malignancies in a phase I-Ib/II open-label multicenter study (NCT02608268). TSR-022 is another anti-TIM-3 monoclonal antibody and its safety and efficacy are assessed alone in patients with advanced solid tumors (NCT02817633) or in combination with TSR-042 (anti-PD-1 antibody) (NCT03307785). Notably, a phase II trial studying the effect of TSR-022 with TSR-042 in the treatment of patients with locally advanced or metastatic liver cancer is recruiting and results are pending in October 2023 (NCT03680508). There are also various TIM-3 inhibitors studied in the phase I trials, including Sym023 (NCT03489343), BMS986258 (NCT3446040), and RO7121661 (NCT03708328).

In the mice model of nephrotoxic serum nephritis, TIM-3 was found up-regulated in kidneys and exerted a protective role. Administration of the anti-TIM-3 antibody aggravated nephritis as shown by significantly increased albuminuria, respective

histological changes, and expression of the renal injury molecule lipocalin-2[310]. Paradoxically, in the other mice model of diabetic nephropathy, TIM-3 was found to worsen the disease *via* the NF-κB/TNF-α pathway, and its performance in macrophage worsened podocyte injury both *in vivo* and *in vitro* studies[311]. Given that limited data is available, its exact role in the development of renal diseases remains unclear. Currently, the renal side effects of anti-TIM-3 antibodies in humans are still unknown. The renal safety profile from the clinical trials is still awaited.

Antivirals use during HCC treatment

Concomitant antiviral therapy is common during HCC treatment, especially the use of anti-HBV nucleoside or nucleotide analogues (NUCs). NUCs therapy could suppress HBV viral replication, achieve biochemical remission, and ameliorate liver inflammation[312-315]. In addition, NUCs therapy could reduce the incidence of liver decompensation, particularly in HCC patients undergoing LT on immunosuppressant and TACE which are prone to cause HBV reactivation or flare[316-319]. Though there is emerging evidence shows NUCs could decrease HCC incidence and recurrence[315, 320-325], the extent to which NUCs therapy may reduce the risk for HCC has been debated[326-328]. Recent mainstay therapies for HBV include NUCs with high potency and high genetic barriers, such as entecavir (ETV), tenofovir disoproxil fumarate (TDF), and tenofovir alafenamide (TAF). However, when using in patients with renal dysfunction, there are special considerations that need to be watched.

ETV belongs to a nucleoside analogue with high potency. However, it has lower efficacy if lamivudine resistance presents previously[329]. Relatively safe renal safety profiles were reported both in rats and humans[330-333]. Since ETV is eliminated primarily from kidneys, renal dose adjustment is needed in patients with CCR less than 50 mL/min to avoid over-exposure to this drug[334,335].

TDF, a nucleotide analogue, is a prodrug of tenofovir that is absorbed from the intestine and cleaved to release tenofovir, which is then phosphorylated inside hepatocytes to form active tenofovir diphosphate targeting viral reverse transcriptase [336]. The adverse effects of long-term use include elevated creatinine, Fanconi syndrome, and osteoporosis[337,338]. TDF is also eliminated by the kidneys in the majority and is not suggested in patients with CCR less than 50 mL/min by some society guidelines because of its nephrotoxicity to proximal renal tubules[339]. Furthermore, when using in the scenario of HCC treatment, renal functions of patients receiving repeated computed tomography exams should be closely followed in case of deterioration[340].

TAF is the other novel prodrug of tenofovir. In an *in vitro* study, TAF resulted in high levels of the pharmacologically active metabolite tenofovir diphosphate than TDF [341]. A recent study showed comparable viral suppression and serologic response between TAF and TDF in non-cirrhotic HBV patients[342]. Besides, TAF has no proximal renal transporter-dependent cytotoxicity, which may lead to an improved renal safety profile[343]. Nevertheless, there was insufficient data in patients whose CCR below 15mL/min not receiving chronic hemodialysis and thus TAF is not recommended in this patient group[344].

CONCLUSION

The HCC patient population highly overlaps with those for CKD and ESRD. This article summarized the possible causes of the high comorbidity of HCC and renal dysfunction (Figure 1), including the possible mechanisms of CKD causing HCC, the pathophysiology of HCC affecting renal function, and the common risk factors shared by both HCC and CKD (Table 1). Both CKD and ESRD have been reported to negatively affect the prognosis of HCC. The article then adopted the well-known BCLC guidelines as a template (Figure 2) to discuss the indications for each stage of HCC treatment, the treatment-related adverse renal effects, and the concerns that are specific to patients with pre-existing renal dysfunction in the application of aggressive treatments such as liver resection, SLKT, RFA, and TACE, and in the use of the latest systemic target and immunotherapy approaches among the CKD and ESRD population. This article provides a comprehensive review of HCC patients with renal dysfunction from disease onset to treatment; the findings are expected to aid clinicians and scholars.

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MINIREVIEWS

Abdominal and gastrointestinal manifestations in COVID-19 patients: Is imaging useful?

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Abstract

Coronavirus disease 2019 (COVID-19) can be considered a systemic disease with a specific tropism for the vascular system, in which the alterations of the microcirculation have an important pathogenetic role. The lungs are the main organ involved in COVID-19, and severe progressive respiratory failure is the leading cause of death in the affected patients; however, many other organs can be involved with variable clinical manifestations. Concerning abdominal manifestations, the gastrointestinal tract and the hepatobiliary system are mainly affected, although the pancreas, urinary tract and spleen may also be involved. The most common gastrointestinal symptoms are loss of appetite, followed by nausea and vomiting, diarrhea and abdominal pain. Gastrointestinal imaging findings include bowel wall thickening, sometimes associated with hyperemia and mesenteric thickening, fluid-filled segments of the large bowel and rarely intestinal pneumatosis and ischemia. Hepatic involvement manifests as an increase in the enzymatic levels of alanine aminotransferase, aspartate aminotransferase, serum bilirubin and γ-glutamyl transferase with clinical manifestations in most cases mild and transient. The most frequent radiological features are hepatic steatosis, biliary sludge and gallstones. Edematous acute pancreatitis, kidney infarct and acute kidney injury from acute tubular necrosis have been described more rarely in COVID-19. Lastly, splenic involvement is characterized by splenomegaly and by the development of solitary or multifocal splenic infarcts with classic wedge-

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shaped or even rounded morphology, with irregular or smooth profiles. In summary, the abdominal radiological findings of COVID-19 are nonspecific and with poor pathological correlation reported in the literature. Ultrasound and particularly computed tomography with multiphasic acquisition are the diagnostic methods mainly utilized in COVID-19 patients with abdominal clinical symptoms and signs. Although radiological signs are not specific of abdominal and gastrointestinal involvement, the diagnostic imaging modalities and in particular computed tomography are helpful for the clinician in the management, evaluation of the severity and evolution of the COVID-19 patients.

Key Words: COVID-19; SARS-CoV-2; Abdominal findings; Gastrointestinal findings; Computed tomography; Ultrasound

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Core Tip: Coronavirus disease 2019 (COVID-19) pulmonary involvement has been extensively reported in the literature. Nowadays, a series of published data highlight how COVID-19 is a systemic disease affecting many other organs. Abdominal and gastrointestinal clinical manifestations have been more recently investigated with imaging modalities such as ultrasound and computed tomography. The aim of this review is to report the most common imaging features of abdominal and gastrointestinal involvement and the possible role of ultrasound and particularly computed tomography in the management, evaluation of the severity and evolution of COVID-19 patients.

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INTRODUCTION

Severe acute respiratory syndrome by severe acute respiratory syndrome corona-virus 2 (SARS-CoV-2) was first recognized in December 2019 in Wuhan (Hubei province, China), but its origin is still unknown and debated. Since then, the disease spread worldwide, leading the World Health Organization to declare a global pandemic on March 11, 2020[1]. More than 21 million confirmed cases of coronavirus disease 2019 (COVID-19) have been reported on all continents except Antarctica, and the incidence is steadily increasing[2]. In all territories affected by the COVID-19 pandemic, the diagnostic tests, the workload of the intensive care units and the initiation of mitigation strategies, such as the restriction of interpersonal contacts, have been established and increased over time[3]. Men seem to be disproportionately more commonly affected by a SARS-CoV-2 infection, and the hospital mortality rate among males is significantly higher than female patients[4]. Main vehicles of transmission of SARS-CoV-2 disease are respiratory droplets being released during coughing, sneezing or conversation between subjects and accumulate on surfaces causing an indirect contamination[5].

SARS-CoV-2 pathogenic agent of this pandemic disease belongs to the Coronaviridae family. Coronaviruses are nonsegmented enveloped RNA viruses with a single-strand linear positive-sense RNA[6]. They are routinely present among animals as well as humans. They are the most common cause of colds, particularly in cats and dogs[7]. Although the origin of the new mutant strain SARS-CoV-2 remains uncertain, it is likely that it originated in a wet market in Wuhan, where animals of all kinds are slaughtered in poor sanitary conditions and their meats are sometimes eaten raw[8].

Six types of coronaviruses causing human disease have been identified: Four of them cause mild respiratory symptoms, whereas the other two, Middle East respiratory syndrome coronavirus and SARS-CoV-1, have previously resulted in epidemics with high mortality rates[9]. SARS-CoV-2 has 80% genomic compatibility

with SARS-CoV-1 and uses the same angiotensin-converting enzyme 2 (ACE2) receptor to enter cells. In fact, ACE2 is an integral membrane protein that seems to be the host cell receptor for SARS-CoV-2, which appears significantly increased in COVID-19 positive patients[10]. ACE2 positive endothelial cells from patients with COVID-19 show significant changes in their morphology, disruption of the intercellular junctions, cell swelling and loss of contact with the basement membrane [11]. The presence of the SARS-CoV-2 virus within endothelial cells suggests that direct viral effect as well as perivascular inflammation may contribute to endothelial damage[11]. This underlines the importance of microcirculation alterations in the pathogenesis and subsequent manifestations at the systemic level[11,12].

Pulmonary involvement in the COVID-19 pandemic is the most known and largely studied because progressive severe respiratory failure represents the leading cause of death in affected patients. Pathological samples of peripheral lung of patients who died from COVID-19 showed a histological pattern of diffuse alveolar damage with perivascular infiltration of T cells[13]. Pulmonary parenchymal tissue also showed typical vascular findings, represented by severe endothelial lesions with the presence of intracellular viruses and interrupted cell membranes[14].

Histological analysis of pulmonary vessels in affected patients showed diffuse thrombosis with microangiopathy; alveolar capillary microthrombi seem to be nine times more frequent as well as the amount of new vessel growth was reported to be 2.7 times higher in patients with COVID-19 in respect to those affected by influenza virus disease[15]. Therefore, three distinctive angiocentric characteristics of COVID-19 were found in the lung: (1) severe endothelial injury associated with the intracellular SARS-CoV-2 virus and rupture of endothelial cell membranes; (2) diffuse vascular thrombosis with microangiopathy and occlusion of the alveolar capillaries; and (3) significant growth of new vessels through an intussusceptive angiogenesis mechanism [13]. Although COVID-19 typically manifests as a respiratory illness and most of the literature described pulmonary signs and symptoms, this disease can affect other anatomical districts and structures, whose clinical manifestations are relatively poorly known. A whole series of signs and symptoms related to systemic manifestations are known to have a certain relevance. In particular, it has been reported that 57% of patients with low severity COVID-19 disease could have reported abdominal discomfort alone or in combination with pulmonary symptomatology[16]. The abdominal clinical manifestations have been related to the gastrointestinal tract and the hepato-biliary-pancreatic system, whereas urinary tract and spleen involvement have been less frequently reported[17].

Our review was aimed to report the current abdominal and gastrointestinal imaging features in COVID-19 patients as well as to define the role of the diagnostic imaging modalities in the abdominal manifestations.

GASTROINTESTINAL TRACT

Abdominal and gastrointestinal signs and symptoms in COVID-19 are being increasingly reported[18]. Indeed, on January 19, 2020, the first known patient of COVID-19 in Washington, United States reported a history of nausea and vomiting in addition to respiratory symptoms[19]. In the literature, it has been reported that series of COVID-19 patients presenting to the emergency room with abdominal pain but without the typical respiratory symptoms of SARS-CoV-2; therefore, the abdominal radiologist was the first to suggest COVID-19 infection because of the typical findings in the lung lower lobes on computed tomography (CT) scans of the abdomen, such as peripheral and subpleural ground-glass opacities[18,20,21] (Figure 1). In this context, some authors have proposed additional CT of the whole chest as part of a CT imaging pathway of acute abdominal pain during the COVID-19 pandemic; this did not get approval because in these patients it was enough just to review the pulmonary bases on abdominal CT scans[22].

Patients with primarily mild gastrointestinal symptoms may not be identified as COVID-19 patients, with serious consequences for themselves and their contacts[18]; in this setting the most common gastrointestinal symptom is loss of appetite, followed by nausea and vomiting, while diarrhea and abdominal pain are the presenting symptoms in only a small percentage of cases[18].

In a retrospective study by Han *et al*[16], patients with gastrointestinal symptoms compared with patients with only respiratory symptoms, tended to have a longer course between symptom onset and viral clearance and took longer to report for medical care, a finding observed in other studies[23]; this suggests that in these

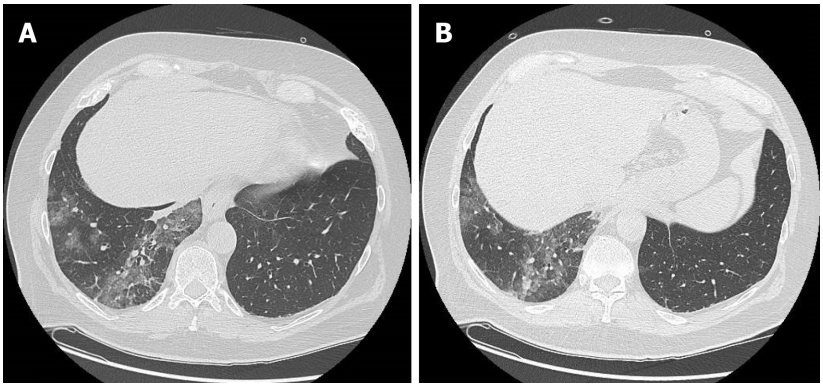


Figure 1 A 63-year-old man who presented to the emergency room for abdominal pain and no significant alterations on abdominal computed tomography. A: Computed tomography scan with pulmonary window at the level of lung inferior lobes (included in the volume acquisition) shows the presence of ground-glass opacities and crazy-paving pattern in the right side; B: Computed tomography scan with pulmonary window at lower levels of lung inferior lobes always exhibited ground-glass opacities and crazy-paving pattern in the right side.

patients the COVID-19 involvement was not initially recognized leading to delayed diagnosis[24]. Moreover, SARS-CoV-2 has been identified in stool samples of a proportion of infected patients[16]; viral replication in both small and large intestine was confirmed by the result of electron microscopy of autopsy biopsy specimen[24, 25]. In particular, an autoptic study on the small intestine of two COVID-19 patients showed endothelitis of the submucosa vessels with mononuclear cell infiltrates within the intima along the lumen of many vessels, besides the evidence of direct viral infection of endothelial cells[11].

The inflammatory response in the gut due to active viral replication is also supported by the evidence of elevated fecal calprotectin concentrations in COVID-19 patients with diarrhea when compared with COVID-19 patients without diarrhea[26].

The radiological alterations of the gastrointestinal system in COVID-19 patients are represented by nonspecific thickening of various regions of the small and large bowel wall[27,28] (Figures 2 and 3), sometimes associated with hyperemia and mesenteric thickening[28] (Figure 4). Goldberg-Stein *et al*[29] retrospectively reported that the most common gastrointestinal symptom in 141 COVID-19 patients was the abdominal pain, present in 73.8% of patients with negative abdominal CT findings and in 53.8% of patients with positive abdominal CT findings; in this series the most commonly reported CT finding was represented by segmental wall thickening of the gastrointestinal tract[29]. In addition, 64% of patients with no positive CT abdominal findings but gastrointestinal symptoms showed suggestive features for COVID-19 pneumonia at the lung bases[29]. This suggests that abdominal symptoms may be present in COVID-19 patients without correlative CT abdominal findings; in fact, in patients with pneumonia it was hypothesized that the abdominal and back pain may be secondary to pleural irritation[30,31].

Tirumani *et al*[32], instead, in a retrospective study identified the incidence of abdominal findings in COVID-19 patients with and without abdominal symptoms and concluded that bowel abnormalities are the most common finding in the abdomen in patients with COVID-19, often without abdominal symptoms and especially regardless of the severity of lung involvement[32]. The most common CT abdominal findings are a fluid-filled colon with no wall thickening (Figures 5 and 6), severe colitis (characterized by a thickened and edematous wall with fat stranding), gastritis and small bowel pneumatosis with portal venous gas[32].

Other minor manifestations are also reported. In a case report, Noda *et al*[33] described a COVID-19 healthy teenager who initially presented with abdominal discomfort. The patient underwent CT scan that demonstrated only isolated mesenteric adenopathy and adjacent fat stranding associated to ground-glass opacities and solid consolidation as well as interlobular septal thickening at lung bases[33]. This case highlights how abdominal findings in COVID-19 patients are not specific and should be suspected given the continued emergence of new manifestations of the disease.

Bhayana *et al*[34] retrospectively analyzed 42 abdominal CT scans performed in COVID-19 patients for abdominal pain or septic status. Colorectal and small bowel wall thickening (defined as single-wall thickness greater than 3 mm in distended intestine and greater than 5 mm in collapsed intestine) were found in 12 out of 42

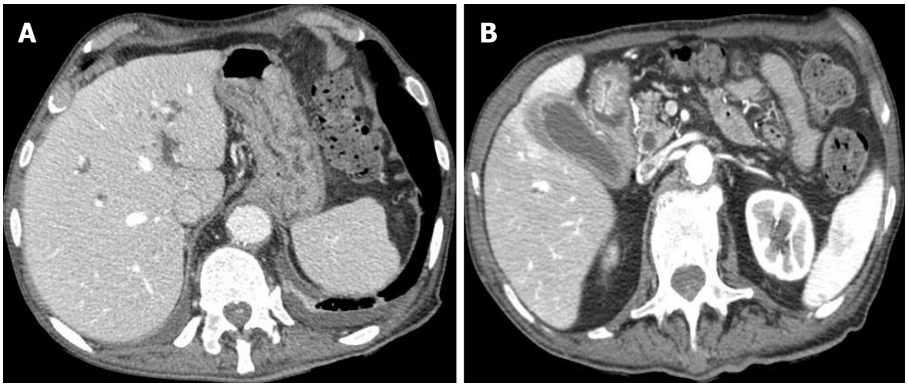


Figure 2 An 83-year-old woman with epigastric pain. A: Abdominal contrast-enhanced portal-venous phase computed tomography showed diffuse thickening of the submucosa of the gastric walls and intrahepatic biliary dilatation; B: Abdominal contrast-enhanced portal-venous phase computed tomography also depicts thickening of the submucosa of the pyloric region and signs of cholecystitis.



Figure 3 A 68-year-old woman with abdominal pain. Abdominal contrast-enhanced portal-venous phase computed tomography image showed circumferential thickening of the submucosa of the right colon that appeared hypodense, in the absence of both significant contrast-enhancement and perivisceral fat stranding.

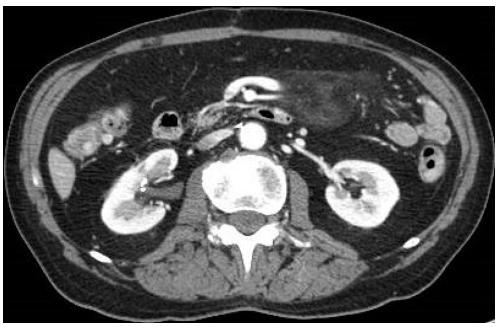


Figure 4 A 74-year-old man with abdominal pain. Abdominal contrast-enhanced portal-venous phase computed tomography image showed well-circumscribed hyperattenuation of the fat surrounding the mesenteric vessels.

abdominal CT, whereas pneumatosis associated to gas in the portal vein and fluid-filled colon (defined as homogeneous, low-attenuation colonic content) were identified in 4 and 18 out of 42 cases, respectively. The remaining 8 patients did not exhibit CT alterations of the gastrointestinal tract[34]. The 4 cases of pneumatosis and portal vein gas underwent exploratory laparotomy; 2 cases had necrotic bowel at surgery with a yellow discoloration of the small bowel in contrast with the usual black or purple color of a necrotic bowel. One patient also underwent a bowel resection demonstrating ischemic enteritis with patchy necrosis and submucosal arterioles containing fibrin thrombi[34].

Bowel ischemia has also been described in COVID-19 patients, particularly in those admitted to the intensive care unit[35], constituting a life-threatening clinical

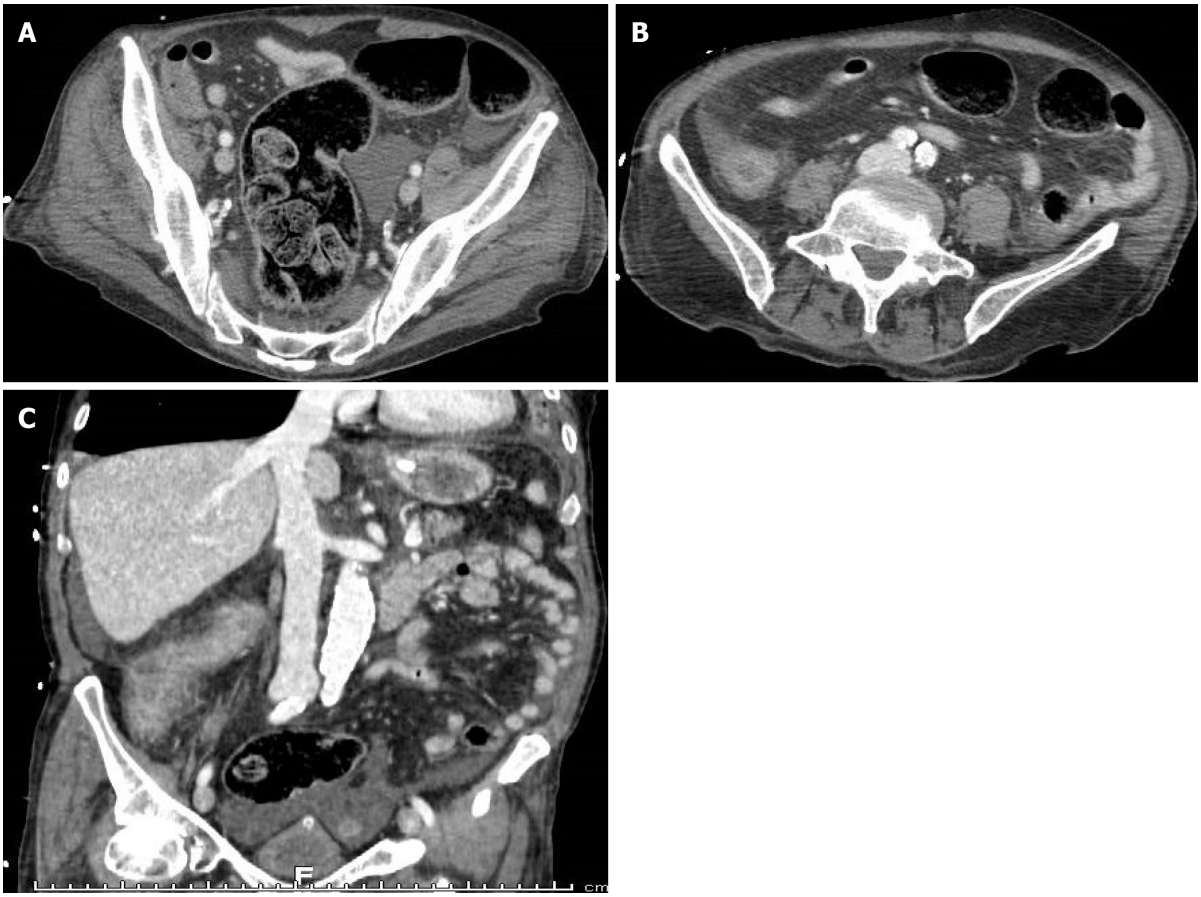


Figure 5 A 73-year-old woman with abdominal pain. A: Contrast-enhanced portal-venous phase computed tomography image of the lower abdomen demonstrated gas distension of the sigma-rectum with evidence of multiple stools inside the lumen; free effusion was also appreciable around the colon; B: Contrast-enhanced portal-venous phase computed tomography image of the abdomen showed distention of the left bowel and hyperemic thickened walls (particularly affecting the mucosa) of the right colon with perivisceral fat suffusion; C: Coronal multiplanar reconstruction from contrast-enhanced computed tomography well demonstrated these findings.



Figure 6 A 69-year-old woman with diarrhea. Contrast-enhanced portal-venous phase computed tomography image of the abdomen showed evidence of fluid-filled distension of the large bowel, particularly of the sigma and rectum, without evidence of parietal thickening. Free effusion was also present in the abdomen and between the intestinal loops with associated diffuse imbibition of the subcutaneous soft tissues.

emergency[9]. It is well known that the hypercoagulable state induced by COVID-19 results in micro- and macrovascular complications[36]. The microvascular complications are detected in the early stages of the disease, while the macrovascular ones are more typically observed in severely ill patients[37]. Anyway, thromboembolic disease within the mesenteric vascular system is not frequently identified on CT imaging[35].

Revzin *et al*[38] divided the COVID-19 related bowel ischemia into early, intermediate and late presentations[38]. On CT images the early phase shows contracted gasless bowel that may transform into dilated gas-filled bowel with a paper-thin bowel wall in the intermediate phase. The CT findings of the late phase

include intestinal wall pneumatosis, absence of mucosal enhancement and luminal dilatation. It is important to note that these phases reflect those of a classic intestinal ischemia regardless of etiology[39] and that the presence of pneumatosis intestinalis suggests bowel ischemia[36], but its presence must be interpreted with caution because it may be secondary to mechanical ventilation in patients with severe COVID-19[40] (Figure 7).

Actually, the exact pathological mechanism of intestinal ischemic disorders is not clearly known. In a letter to the editor, Parry *et al*[41] reported four possible mechanisms that work alone or in varying combination: coagulation disorder, elevated levels of von Willebrand Factor, expression of ACE2 on enterocytes of the small bowel and shock/hemodynamic compromise associated with COVID-19 pneumonia[41].

COVID-19 patients may be in a state of hypercoagulability induced by a systemic inflammatory state, endothelial activation, hypoxia and immobilization. These could lead to mesenteric microvascular thrombosis without involvement of the large mesenteric vessels, resulting in a condition of thrombosis in situ rather than an embolic event[41].

Elevated levels of von Willebrand factor have been reported in severe COVID-19 patients[42]. von Willebrand factor is released from endothelium in response to damage caused by SARS-CoV-2 and consequent endothelium dysfunction and vascular thrombosis[43].

Regarding the last two points, the enterocytes express ACE2, the target receptor for SARS-CoV-2 with intestinal tropism and direct bowel damage. Lastly, shock or hemodynamic compromise, which is commonly associated with severe COVID-19 pneumonia, may lead to a non-occlusive mesenteric ischemia[42].

LIVER, BILIARY TRACT AND PANCREAS

The liver is the second most frequently injured organ after the lung in COVID-19[44]. The mechanism of liver damage is probably due to a series of events that can occur simultaneously. Direct cytopathic effect of SARS-CoV-2, indirect damage from systemic inflammation, drug hepatotoxicity[44] and hypoxic alterations related to ventilation have been mainly described[45]. SARS-CoV-2 causes direct liver damage because the ACE2 receptor is widely expressed in the liver, more on cholangiocytes than on hepatocytes[46]. The virus alters the barrier and bile acid transport functions of cholangiocytes through the dysregulation of genes involved in tight junction formation and bile acid transport[47]. Drugs commonly used during SARS-CoV-2 infection causing liver toxicity include remdesivir, tocilizumab, chloroquine, hydroxychloroquine and azithromycin[48]. The immune-mediated cytokine storm also participates in the damage; in fact, we have a marked activation of inflammatory markers, including abnormal levels of C-reactive protein, lymphocytes, neutrophils and cytokines, in particular interleukin-6. The control of cytokine dysregulation at an early stage could be useful to slow down the progression of the disease[45]. In addition, respiratory-induced hypoxia can cause elevated serum aminotransferase concentrations, a laboratory marker of liver injury[49]. The combination of these events determines a generalized coagulopathy state that determines an alteration of the microcirculation with microthrombosis within the hepatic sinusoids[20]. As a support to this etiopathogenetic theory, liver autopsy results by Medeiros *et al*[50] show periportal necrosis, lymphocytic infiltration of the sinusoids, dense infiltration of the gate by abnormally small lymphocytes, central venous thrombosis and cirrhotic alterations with fibrosis in a retrospective series of 316 patients[50].

Liver injury usually manifests as an increase in enzyme levels. Current literature data show that 14.8%-53.0% of COVID-19 patients have abnormal levels of alanine aminotransferase and aspartate aminotransferase and a slight increase in serum bilirubin levels during the course of the disease[16]. Phipps *et al*[51] found that an alanine aminotransferase spike was significantly associated with clinical outcome[51]. In respect to mild to moderate or no liver injury, patients with severe liver injury have a higher rate of intubation and renal replacement therapy. Additionally, approximately 50% of COVID-19 patients had increased levels of γ-glutamyl transferase[52].

To the best of our knowledge, hepatic manifestations of COVID-19 were in most cases mild and transient. Despite this, there is an ever increasing number of subjects with severe hepatic manifestations that are very often associated with the pulmonary ones[53].

For the management of COVID-19 patients with liver injury, the American Association for the Study of Liver Diseases provides recommendations, highlighting

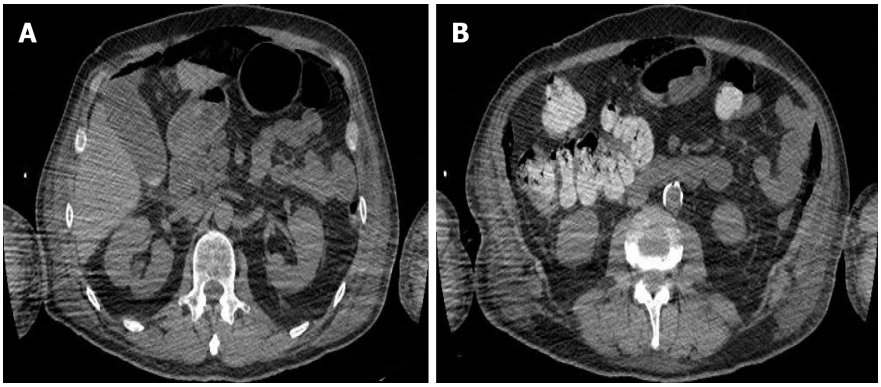


Figure 7 A 65-year-old man with abdominal pain and severe pulmonary involvement. A: Unenhanced computed tomography scan of the upper abdomen showed signs of pneumoperitoneum secondary to mechanical ventilation and cholelithiasis; B: The presence of gas within the peritoneal cavity was also appreciable at lower levels of the abdomen on unenhanced computed tomography scan.

that there are no contraindications to the use of drugs such as remdesivir, tocilizumab, chloroquine, hydroxychloroquine and azithromycin unless alanine aminotransferase or aspartate aminotransferase are no more than five times the upper normal limit[48].

A systematic review and meta-analysis of international data on the hepatic manifestations of COVID-19 was performed by The American Gastroenterological Association Institute[54]. Among COVID-19 patients with liver injury, more than 60% of patients had mild hepatic injury (1 time upper normal limit to 5 times upper normal limit)[51]. It should be noted that previous liver disease and underlying liver function may have influenced the results. In fact, some patients may have abnormal liver function prior to SARS-CoV-2 infection, such as nonalcoholic fatty liver disease or chronic hepatitis B[55]. Moreover, there is a higher prevalence of hepatic steatosis that is probably due to the known association between infection and obesity[50].

Hepatic steatosis is a very frequent and nonspecific finding of COVID-19 patients, which has been identified with both ultrasound (US) and CT. The findings of hepatic steatosis are the same as those found in non-COVID-19 patients. On US we can observe the typical "bright liver" that is characterized by an increase in echogenicity compared to the renal cortex or spleen (in the case of renal pathology), loss of physiological hyperechogenicity of the portal branches walls and posterior attenuation of the ultrasonic beam with failure to visualize the diaphragm (Figure 8). On the other hand, diffuse liver hypodensity is the typical sign on CT scans. In the presence of a slight steatosis a hepatic attenuation of less than 10 HU compared to the density of the spleen is observed, whereas in moderate/severe forms hepatic attenuation is less than 40 HU compared to the spleen[56] (Figure 9).

Ji *et al*[57] found that COVID-19 patients with nonalcoholic fatty liver disease were more likely to have liver damage and disease progression than patients without nonalcoholic fatty liver disease[57]. Singh *et al*[58] studied the impact of pre-existing liver disease on outcomes in a large cohort of COVID-19 patients and found that the risk of hospitalization and death in patients with pre-existing liver disease was significantly higher[58].

Further, in 54% of patients with COVID-19, biliary sludge and gallstones were found and were closely related to an increase in cholestasis indexes[59], values that appear to be markedly increased in comparison with the incidence in the general population (about 10%-20%)[38]. US is considered the gold standard for detecting gallstones. It allows the evaluation of macro- and microlithiasis, sludge and cholesterol deposits as well as the structural evaluation of the gallbladder[60]. The cholesterol deposition along the gallbladder walls appears as hyperechogenic spots with the typical "comet sign" whereas the biliary sludge as sediment in the declivous portion of the gallbladder lumen[60] (Figure 10). Although the role of CT in the evaluation of biliary lithiasis is marginal compared to US in the normal patient, this technique easily allows the visualization of hyperdense calcium stones and hypodense cholesterol stones in COVID-19 patients because US is not easily performed in the most compromised patients[61].

Pancreatic involvement has also been described in COVID-19 patients. As for other districts, the pathogenetic mechanism is not yet clear and could be the result of direct (cytopathic effect of the virus) or indirect (immune-mediated storm) mechanisms[62].

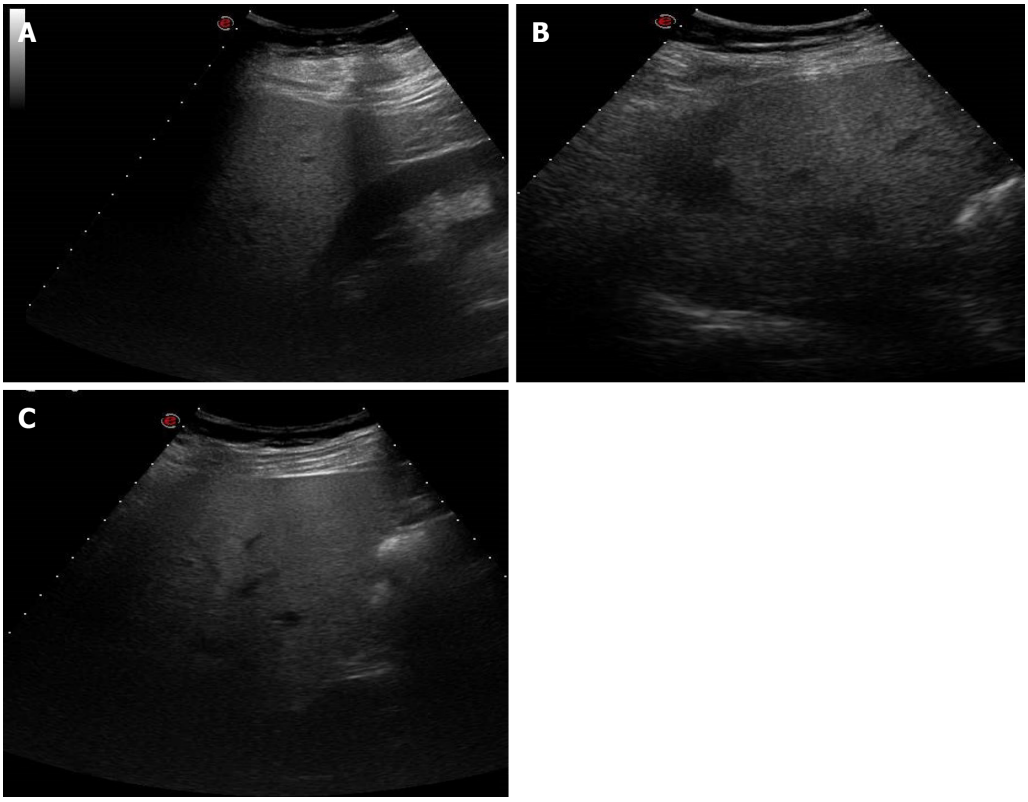


Figure 8 A 38-year-old man with abdominal discomfort. A: Abdominal ultrasound image demonstrated hepatic steatosis as an increase in echogenicity compared to the renal cortex; B: Abdominal ultrasound image demonstrated hepatic steatosis as loss of physiological hyperechogenicity of the wall of the portal branches; C: Abdominal ultrasound image demonstrated hepatic steatosis as posterior attenuation of the ultrasonic beam with failure to visualize the diaphragm.



Figure 9 A 46-year-old woman with abdominal discomfort. Unenhanced computed tomography image showed increased liver hypodensity compared to the spleen, with attenuation value less than 40 HU.

The presence of the ACE2 receptor on the cells of the pancreatic islets and exocrine glands allows the penetration of the virus thus determining the manifestations. In a series of 52 patients with COVID-19 pneumonia by Wang *et al*[63], 17% of them had pancreatic injury with elevated blood glucose levels. These results show potential mild pancreatic injury patterns in patients with COVID-19 pneumonia with no severe pancreatitis as a common manifestation[63]. However, some cases of pancreatitis have been reported in the literature[64]; in 64 patients with severe COVID-19 Liu *et al*[62] reported that 17.9% and 16.4% had increased amylase and lipase levels, respectively [62]. Thirteen out of 64 patients were examined with CT scans; of these only 5 patients showed radiological pancreatic alterations. It is worth underlining that most of these cases have been reported in moderate or severe disease. This seemingly suggests that the pathophysiology of pancreatitis could be based on the systemic inflammatory response rather than a direct histopathological effect[62].



Figure 10 A 46-year-old woman with right hypochondrium pain. Abdominal ultrasound showed an enlarged gallbladder containing deposit of biliary sludge in the infundibular region.

The radiological signs of pancreatitis in COVID-19 patients were the same as we find in classic acute pancreatitis: Focal or diffuse parenchymal enlargement and density changes due to edema with indistinct pancreatic margins and stranding of retroperitoneal fat[65] (Figure 11). More rarely, necrotic-hemorrhagic forms of pancreatitis have been described[65]. The presence of gas (emphysematous pancreatitis) and the presence of calcifications as a sign of acute on chronic pancreatitis have also been reported[66].

URINARY TRACT

There is increasing evidence that acute kidney injury (AKI) develops commonly in COVID-19 patients[67] because it affects approximately 20%-40% of patients admitted to the hospital and particularly to the intensive care unit in Europe and in the United States[68,69]. COVID-19 patients developing AKI, in conjunction with respiratory symptoms, have a poor prognosis, with a 35% reported mortality[70].

Possible causes of COVID-19-related AKI include dehydration, hypoperfusion from myocardial dysfunction, immune response dysregulation (cytokine storm) or direct kidney endothelial damage by SARS-CoV-2[69], which manifests clinically and pathologically by the development of acute tubular necrosis, interstitial inflammation, podocytopathy, microangiopathy and collapsing glomerulopathy[69].

In a postmortem renal histopathological analysis of 26 COVID-19 patients, Su *et al* [71] reported a histopathological finding of acute tubular necrosis due to endothelial damage causing microvascular lumen occlusion. The authors hypothesized that their results had been dependent on the possible kidney cells' infections with SARS-CoV-2 [71].

In this setting the imaging modality of choice is US. It is a bedside examination that can show increased cortical echogenicity or heterogeneity and loss of corticomedullary differentiation in patients with COVID-19 and AKI[72]. In cases of renal infarction, heterogeneity and hypoperfusion of the renal parenchyma and wedge-shaped areas of decreased perfusion and/or enhancement may be visualized on US and contrast-enhanced CT and may be multifocal, involving both kidneys[72] (Figure 12). It is important to note that if renal function is impaired, the use of iodinated contrast material is not recommended, thus making US the imaging modality of choice in the evaluation of COVID-19 patients with suspected renal vascular injury.

In a retrospective study, Hectors *et al*[73] found that cortex-to-aorta enhancement index (*i.e.* the ratio of renal cortical density to aorta density on contrast-enhanced CT) at the time of COVID-19 diagnosis was significantly reduced in patients who ultimately developed AKI. The authors suggest that reduced renal perfusion in COVID-19 precedes full-fledged AKI[73].

In another retrospective study, Huang *et al*[74] highlighted the usefulness of non-contrast CT on the assessment of renal impairment associated with COVID-19, featuring as perinephric fat stranding (PFS) and decreased renal parenchymal density. PFS corresponds to the thickening of perinephric bridging septa, which are fibrous lamellae that divide the perinephric space into multiple compartments, limiting the distribution of fluids, such as urine, pus and blood[75]. In this study, patients with PFS

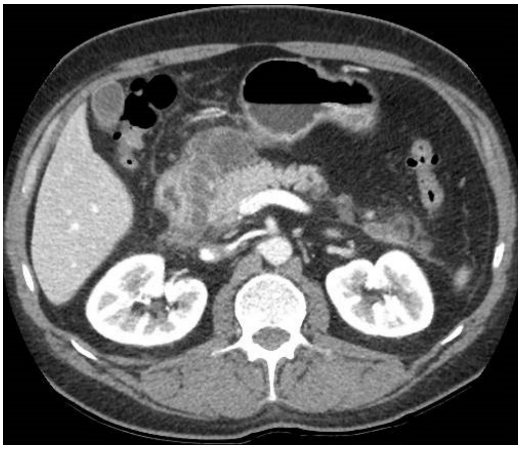


Figure 11 A 60-year-old man with abdominal pain and increased amylase and lipase levels. Abdominal contrast-enhanced portal-venous phase computed tomography image showed fluid collections at the level of the pancreatic head and isthmic region and thickening of the left anterior pararenal fascia and perivisceral fat.

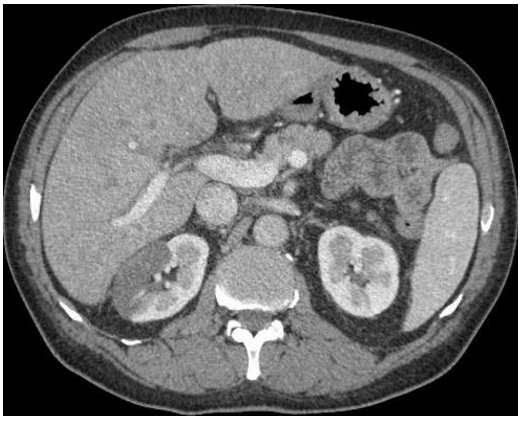


Figure 12 A 69-year-old man with hematuria and right abdominal pain. Abdominal contrast-enhanced portal-venous phase computed tomography image depicted wedge-shaped parenchymal defects that involved both the renal cortex and medulla with extension to the capsular surface, suggesting a renal infarct.

showed elevated serum creatinine levels, higher than that of the group of COVID-19 patients without PFS. Always in this study renal parenchymal attenuation in COVID-19 patients decreased. Moreover, patients with PFS showed a greater attenuation decrease, whereas patients without PFS presented a smaller decrease[75]. Therefore, the authors propose the use of PFS and renal parenchymal attenuation on non-contrast CT as a qualitative indicator for detecting renal damage associated with COVID-19.

Currently, no report has demonstrated the association between COVID-19 and urolithiasis[76], despite the nonsteroidal anti-inflammatory drugs (commonly used in stone-related colic pain) increase ACE2. This might increase the risk of developing severe and fatal COVID-19[77]. Nevertheless, the United States Food and Drug Administration recently announced that there was not enough scientific evidence connecting the use nonsteroidal anti-inflammatory drugs with worsening COVID-19 symptoms[78].

SPLEEN

Splenic injury is commonly encountered in COVID-19 patients. Red pulp cells and endothelial cells of blood vessels show an abundance of ACE2 receptors on their surfaces, so COVID-19 can directly target macrophages and dendritic cells in the spleen[79]. Autopsies in patients who died from COVID-19 exhibited splenic parenchymal congestion, hemorrhage and lack of lymphoid follicles with splenic parenchymal atrophy[79].

Splenic injuries are characterized by splenomegaly and by the development of either solitary or multifocal splenic infarcts[79]. On US examination splenic infarcts are hypoechoic compared to the remaining splenic parenchyma, although in the acute phases they may appear isoechoic and therefore difficult to identify. The morphology of the infarcts can vary as they may be classically wedge-shaped but also rounded, with irregular or smooth profiles. Over time, phenomena of contraction and scarring can develop, and in this case, they will appear as a hyperechoic region with retraction of the splenic capsule. In case of liquefaction, the area may be rounded and anechoic (splenic pseudocyst)[80]. CT is often considered the imaging method of choice, with the best visualization of infarcts during the portal-venous phase, to avoid confusing the heterogeneous improvement normally seen during the arterial phase. The CT imaging characteristics may vary with the stage of the infarct. In the hyperacute phase, there are areas of greater mottled attenuation, which represent areas of a hemorrhagic infarction typically with a wedge-shaped morphology and peripheral localization[81]. In the chronic phase, splenic infarcts may no longer be seen, or more commonly they may undergo fibrotic contraction with consequent loss of volume of the splenic parenchyma (Figure 13). As mentioned for the US, if the infarct liquefies, a cystic lesion can be left with central fluid density[81].

RADIOLOGICAL COMMENTS

As pointed out by the data of the most recent literature, COVID-19 is a systemic disease and not just a pulmonary disease, with a specific tropism for the vascular system. In fact, many other organs besides the lung can be involved by SARS-CoV-2 and thus the clinical manifestations can be variable. The gastrointestinal tract and hepato-biliary system are mainly affected, although the pancreas, urinary tract and spleen may also be involved.

The abdominal radiological findings are nonspecific and with poor pathological correlation reported in the literature. The correct use of imaging modalities in the management of these patients can be extremely helpful for the clinicians. US and particularly CT with multiphasic acquisition are the diagnostic methods mainly utilized in the COVID-19 patients with abdominal clinical symptoms and signs.

Although US can be quickly performed at the bedside and in intensive care units, this diagnostic modality is not always able to provide us reliable information in critically ill, unprepared and uncooperative patients. It also has the problem of exposing healthcare personnel to a consistent risk of infection and this limits its routine use in this setting. On the basis of the published data, US is mainly used for the hepatobiliary system, kidney and spleen evaluation, less for the pancreas and almost not utilized for gastrointestinal system assessment.

CT plays a pivotal role in identifying the signs of abdominal involvement, particularly for the gastrointestinal system, and is extremely useful for the evaluation of the vascular and parenchymal structures. Thanks to this method we can establish the severity and the evolution of the disease in COVID-19 patients. CT study should be performed at the baseline and after iodinated contrast medium injection in the various phases, paying attention to the arterial phase for ischemic lesions of the intestine and kidneys, in the portal-venous phase for wall thickening in colitis or for signs of acute pancreatitis or splenic infarcts and in the late phase for the evaluation of renal nephritis or ischemia.

Actually, magnetic resonance imaging has no defined role, and no significant experience is reported to our best knowledge. This is probably due to the fact that COVID-19 patients have difficulty undergoing magnetic resonance examination and the serious problems of management of these patients.

What is desirable in the future is that the abdominal and gastrointestinal manifestations identified by imaging may have a pathological correspondence in order to further clarify the etiopathogenetic mechanisms of the damage caused by COVID-19. Furthermore, larger series of COVID-19 patients studied with imaging methods could help us to identify the most characteristic signs of abdominal and gastrointestinal involvement. In this setting, magnetic resonance imaging could be particularly useful for the evaluation of the hepato- biliary system, pancreas and kidney.

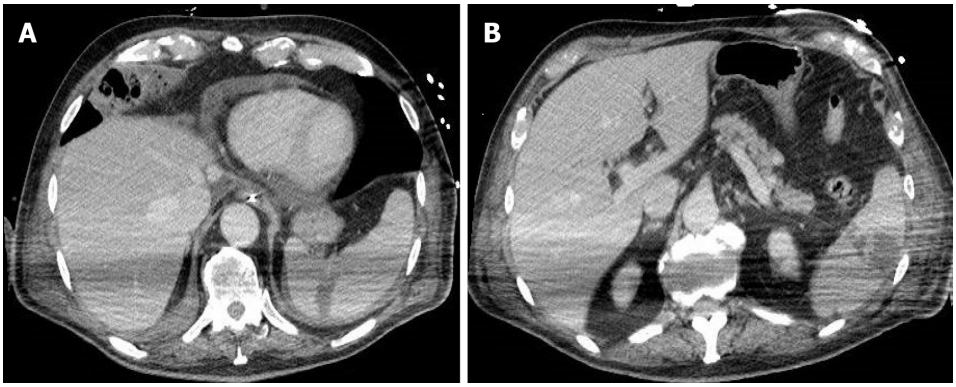


Figure 13 A 77-year-old man with abdominal tenderness. A: Contrast-enhanced portal-venous phase computed tomography image of the abdomen demonstrated a wedge-shaped low-attenuation area at the level of the spleen, typical of infarction. Pericardial effusion was also present; B: A further rounded low-attenuation area with peripheral localization was present in a lower portion of the spleen on contrast-enhanced portal-venous phase computed tomography scan.

CONCLUSION

Although radiological signs are not specific of abdominal and gastrointestinal involvement, the diagnostic imaging modalities and in particular CT are helpful for the clinician in the management, evaluation of the severity and evolution of COVID-19 patients.

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Inflammatory effect on the gastrointestinal system associated with COVID-19

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Abstract

The severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) that causes coronavirus disease-2019 (COVID-19) has provoked a global pandemic, mainly affecting the respiratory tract; however, a percentage of infected individuals can develop gastrointestinal (GI) symptoms. Some studies describe the development of GI symptoms and how they affect the progression of COVID-19. In this review, we summarize the main mechanisms associated with gut damage during infection by SARS-CoV-2 as well as other organs such as the liver and pancreas. Not only are host factors associated with severe COVID-19 but intestinal microbiota dysbiosis is also observed in patients with severe disease.

Key Words: SARS-CoV-2; Gastrointestinal symptoms; COVID-19; Gastrointestinal system

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Core Tip: Coronavirus disease-2019 (COVID-19) affects not only the respiratory systems but also gastrointestinal (GI) system and function of others organs. Until now, the mechanism of infection that severe acute respiratory syndrome, coronavirus 2 uses is not fully known. GI symptoms are rare but had great relevance in the severity of disease. We summarize the main known mechanisms that are associated with intestinal

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damage, and the knowledge that is had about the impact of COVID-19 on the liver and pancreas.

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INTRODUCTION

Coronaviruses are a family of viruses that cause illnesses such as the common cold, severe acute respiratory syndrome, coronavirus 2 (SARS-CoV-2), and Middle East respiratory syndrome (MERS)[1]. SARS-CoV-2 is the etiologic agent of coronavirus disease 2019 (COVID-19), designated as a pandemic by the World Health Organization on March 11, 2020. Up to January 1st, 2021, COVID-19 has caused globally over 85 million cases[2]. The impact that COVID-19 has had worldwide on health and the economy has been devastating since the number of deaths continues, partly because neither we fully understand the disease nor its transmission. Moreover, there are increasing long-term complications and sequelae after COVID-19 in some people[3,4].

The respiratory tract is the main entry route reported, and the transmission mechanism is *via* large droplets containing a high enough viral load. The virus is not motile by itself and depends on its rotational diffusivity to align its proteins (organized in hollow spikes called "peplomers") to its targets during the infection process[5]. Infected people in most cases do not develop symptoms (asymptomatic) or have mild symptoms such as fever, dry cough, fatigue, sore throat and/or headache, conjunctivitis, nausea, vomiting, skin rashes, and dysgeusia; which appear 2-14 d after being exposed to the virus[6]. It has been reported that the survival time of SARS-CoV-2 in aerosol form is 4 h, as the virus becomes inactive at 60°C. Propagation of the droplets in the air depends on the ventilation systems of the area where an infected person is spreading the virus while breathing without using personal protection equipment[7].

Additionally, gastrointestinal (GI) symptoms such as diarrhea, nausea, and vomiting have been reported[8], yet this seems to affect only about 1%–3.8% of the studied patients[9]. Nevertheless, the exact molecular mechanism with which SARS-CoV-2 produces GI damage is still unknown. Therefore, this review aims to describe the effect that SARS-CoV-2 produces in the GI tract.

MECHANISM ASSOCIATED WITH COVID-19 INFECTION IN THE GI SYSTEM

SARS-CoV-2 clinical manifestations include GI effects; however, there is insufficient research on the mechanisms that allow digestive colonization by a respiratory virus. With over 80% resemblance between SARS and SARS-CoV-2[8], several studies have shown tropism for the GI tract, as SARS-CoV-2 RNA was detected in stool specimens from COVID-19 patients with diarrhea, suggesting that it can be transmitted by the fecal-oral route[10].

The viral nucleocapsid protein of SARS-CoV-2 has been found in the GI lumen in the esophagus, stomach, duodenum, and the rectal glandular epithelial cells, suggesting this receptor as the entry point of the SARS-CoV-2 virus in the intestinal tract[10-12]. Also, the expression of angiotensin-converting enzyme 2 (ACE2) protein on glandular cells of gastric, duodenal, rectal epithelia (abundantly expression), and esophageal mucosa (less expression) was demonstrated, supporting the entry of SARS-CoV-2 into the host cells by immunofluorescent technique[13].

ACE2 is a receptor member of the angiotensin-converting enzyme (ACE) family of dipeptidyl-carboxypeptidase and is highly homologous to ACE1, which plays an important role in SARS-CoV-2 infection, through a high-affinity attachment to ACE2

receptors in human cells[11]. The primary function of ACE2 is the conversion of angiotensin (Ang) 1 to Ang 1-9 and Ang 2 into Ang 1-7. ACE receptors participate in cell proliferation and hypertrophy, inflammatory response, blood pressure, and fluid balance. Specifically, ACE2 has an important role in regulating cardiovascular, renal, and reproductive functions[10]. Besides its high expression in type II alveolar cells (AT2) in the lungs, the GI tract also expresses ACE2 receptor, particularly in the esophageal epithelium, glandular gastric mucosa, enterocytes, and colonocytes. ACE2 is present in the cytoplasm of the epithelial cells of the stomach and intestine and the cilia of glandular epithelial cells[10-12].

Recent studies have shown that SARS-CoV-2 may cause digestive symptoms by direct viral invasion of target cells and by inflammatory injury. The viral infection process involves a series of steps: (1) A direct cytopathic effect; (2) Downregulation of ACE2 expression with an increase of metalloproteinase action; and (3) Dysregulation of the immune system, with over secretion of proinflammatory cytokines[14]. Plasmatic and lymphocytic infiltration with interstitial edema[10]. Figure 1 includes more details about this process.

In general, all coronaviruses encode a surface glycoprotein and spike protein that binds to host cell receptors ACE2 and allows virus entry. The spike (S) protein of SARS-CoV-2 has a high affinity for human ACE2, which is the main entrance into the cell[12]. Furin is an enzyme that can be found on the small bowel, acting as a serine-protease that can divide the viral S-protein into two fragments: S1 and S2, allowing them to interact with ACE2. The separation of the S-spike into S1 and S2 is essential for the attachment of the virion to both the ACE receptor and the cell membrane[15]. S-protein proteases, such as cathepsins, expose the fusion domain to the endosome by acid-dependent proteolytic cleavage. Successful virus entry also requires a cellular serine protease, transmembrane protease serine 2 (TMPRSS2)[16]. TMPRSS2 cleaves the S protein of SARS-CoV-2 on the cell membrane, a process that is critical for the fusion of the viral and cell membranes. Importantly, both ACE2 and TMPRSS2 become highly expressed in the ileum and colon[16-18]. Hoffmann *et al*[19] demonstrated that inhibition of the TMPSSR (the serine protease responsible for splitting the S-spike) blocks the infection of cells by SARS-CoV-2.

After viral entry, RNA translates, and viral proteins become synthesized to form new virions released in the GI tract[13]. Thus, leading the CD4+ T cells to reach the small intestine, causing diarrhea and immune damage[12]. ACE2 participates in regulating intestinal inflammation and diarrhea by being a key enzyme in the renin-angiotensin system. It has been shown that loss of ACE2 leads to Ang 2 accumulation. Moreover, the plasma of COVID-19 patients with severe disease presents higher levels of interleukin (IL)-7, IL-10, granulocyte colony-stimulating factor, and recombinant human interferon-induced protein-10, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1A (MIP1A), and tumor necrosis factor alpha (TNF-α)[18]. The local inflammation could debilitate the epithelial barrier, and these inflammatory changes can be part of cell damage induced by viral replication and spreading [20]. This inflammation process disturbs the gut microbiota promoting the polarization of Th17 in the small intestine, promoting the recruitment of other immune cells such as neutrophils, and inducing intestinal immune damage, diarrhea, and other GI symptoms. Also, intestinal damage and gut microbiota alteration can affect the gut-liver axis by contamination of the liver with host and microbial metabolites through the portal vein[12].

A study by Xiao *et al*[13] showed that among 73 hospitalized patients, 53.42% tested positive for SARS-CoV-2 in the stool. The duration of positive stool results ranged from 1 to 12 d, and 23.29% of patients continued to have positive results in stool after being negative in respiratory samples and presenting positive staining for ACE2 receptor and viral nucleocapsid protein in stomach, duodenum, and rectum biopsies. Raising the question if COVID-19 can be transmitted by the fecal-oral route or transmitted by aerosols generated by toilet fumes has been shown with SARS-CoV-2 [21,22]. A study conducted by Zhang *et al*[23] showed that 39.6% of 140 confirmed COVID-19 patients presented GI symptoms among the most common clinical manifestations[23]. Another study reported that 10.1% of 138 confirmed COVID-19 patients, presented diarrhea and nausea[24], furthermore, a recent report showed that 11.4% of 651 patients showed GI symptoms associated with a more severe presentation of the disease[25]. Nonetheless, patients with SARS and MERS have reported more GI symptoms than COVID-19 patients[26]. There has been a high concern in how COVID-19 can affect the body with pre-existing diseases, inflammatory bowel diseases (IBD), such as Crohn disease and ulcerative colitis.

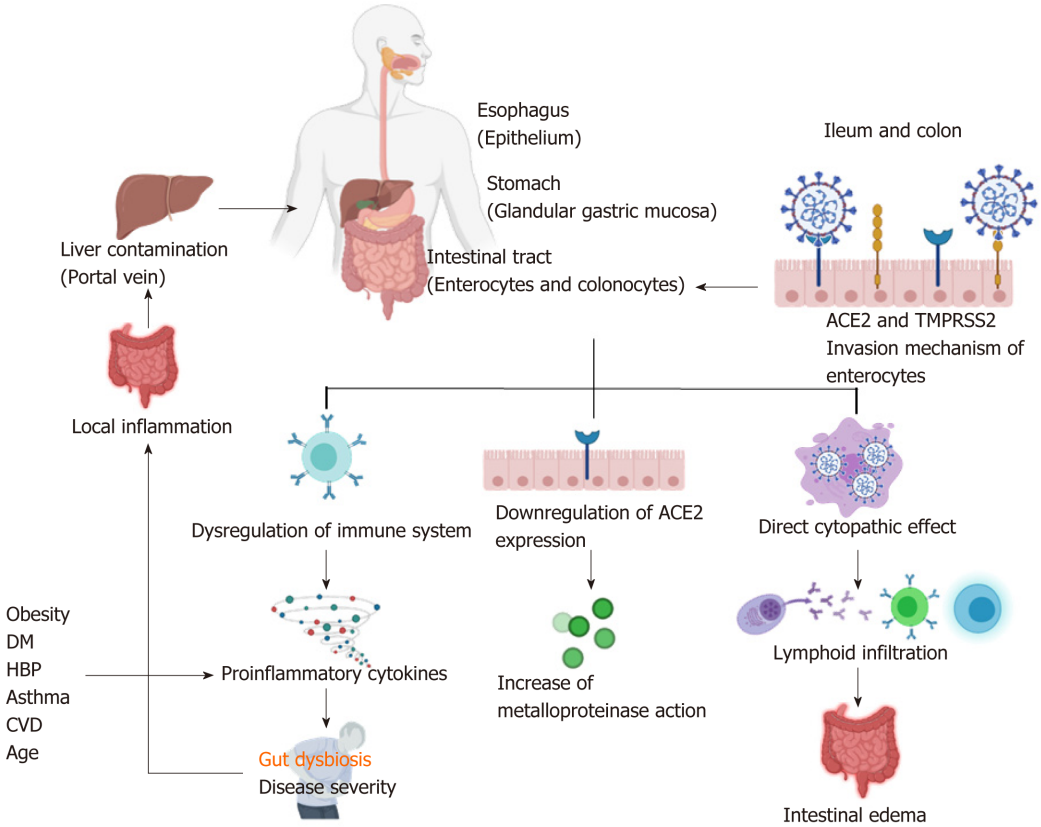


Figure 1 Mechanisms of severe acute respiratory syndrome-coronavirus-2 gastrointestinal infection. The same receptors mediate infections of the gastrointestinal system as in the respiratory system. This situation could begin at the intestinal tract by enterocyte invasion, which possesses angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine 2 receptors recognized by severe acute respiratory syndrome-coronavirus-2. Once in cells, the virus can induce cell death-mediated dysregulation of the immune system by downregulation of ACE2 receptor expression and a direct cytopathic effect. All three mechanisms induce immune dysregulation and increase the inflammation mechanism. Some risk factors that accelerate immune inflammation are obesity, diabetes mellitus, high blood pressure, asthma, cardiovascular disease, and advanced age. Moreover, the virus could enter the liver by the portal vein and induce hepatic failure. ACE2: Angiotensin-converting enzyme 2; TMPRSS2: Transmembrane protease serine 2; DM: Diabetes mellitus; HPB: High blood pressure; CVD: Cardiovascular disease.

A study showed that immunosuppressors modulate the cytokine inflammatory response, thus preventing a more severe manifestation of COVID-19[27]. Also, GI symptoms derived from drug side effects of antibacterials (macrolides, fluoroquinolones, or cephalosporin) and antivirals (chloroquine phosphate, lopinavir, and remdesivir) administered during illness[12].

COVID-19 patients with preexisting comorbidities such as hypertension, asthma, diabetes, cardiovascular problems, and old age, have a higher susceptibility to inflammation. Recent studies have shown that the severity of the clinical course of COVID-19 is related to inflammation and higher levels of proinflammatory cytokines[14]. Studies show that SARS-CoV-2 rapidly activates T cells and induces the release of several inflammatory cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 1 (IL-1), IL-6, monocyte chemoattractant protein-1, and interferon-gamma (IFN-γ). GM-CSF activates CD14+ cells, CD16+ cells, and monocytes, increasing inflammatory cytokine levels, stepping up the inflammatory cascade. This intense immune response causes tissue damage[28]. T cells from peripheral blood in COVID-19 infection present high cytotoxic activity with more cytotoxic granules, granulysin, and perforin, which shows that activated T cells could speed up systemic inflammation[29]. Also, ACE2 expressing cells release proinflammatory cytokines such as MCP-1, tumor growth factor (TGF-1), TNF-α, IL-1, and IL-6 [12].

Recently, COVID-19 intestinal pathogenesis mechanisms have been proposed since SARS-CoV-2 also might interfere with tryptophan absorption. Tryptophan stimulates the mTOR pathway for the production of antimicrobial peptides that maintain gut microbiota homeostasis. This process requires intestinal ACE2 to regulate the expression of neutral amino acid transporters. Tryptophan is absorbed by factors of

the B0AT1/ACE2 transport pathway on the lumen surface of intestinal epithelial cells. When there is not enough niacin or tryptophan intake, there is a high risk of developing pellagra, which eventually develops into colitis. As SARS-CoV-2 infection competes for available ACE2 receptors, it causes tryptophan deficiency and lower production of antimicrobial peptides[16]. COVID-19 murine models showed a deficiency of ACE2 receptors in the colon, which increase susceptibility to inflammation and colitis development due to decreased antimicrobial peptides and the alteration of gut microbiota, finalizing with diarrhea[12,29]. However, this mechanism needs to be proven in humans.

COVID-19 RELATED DAMAGE TO INTESTINAL MICROBIOTA

The human gut microbiota comprises 10^{14} resident microorganisms which include bacteria, *archae*, viruses, and fungi and has a key role in health through its protective function by regulating various host physiological functions, including dietary digestion, and imparting protective immunity against pathogens[30]. The defense mechanism of microbiota induces alpha-defensin, secretory IgA, and some other AMPs (antimicrobial peptides)[31], affecting innate lymphoid cells, but mainly they affect the innate and adaptive immune system by influencing epithelial or macrophage cell receptors, such as toll-like receptors (TLRs) or NOD-like receptors (NLRs). TLRs are involved in normal mucosal immune system development of the intestine, decreasing inflammatory responses and promoting immunological tolerance to the normal microbiota components. NLRs participate in the adjustment of the IL-18 level, the immune response, dysbiosis, and intestinal hyperplasia[32].

Healthy gut microbiota, primarily dominated by *Bifidobacterium* spp., *Faecalibacterium* spp., *Ruminococcus* spp., *and Prevotella* spp. Whom`s alterations in the balance between gut microbiota and the immune system, sometimes collectively called "gut dysbiosis" are associated with infections, inflammations, allergies, colorectal cancer, and autoimmune disease[30]. Studies have suggested that "gut-dysbiosis" might contribute to GI symptoms by SARS-CoV-2 infection, *i.e.*, *Bacteroides dorei, Bacteroides thetaiotaomicron, Bacteroides massiliensis*, and *Bacteroides ovatus*, can downregulate the expression of ACE2 during the hospitalization of COVID-19 patients[33].

Microbial dysbiosis with decreased levels of *Lactobacillus* and *Bifidobacterium* and the abundance of *Clostridium hathewayi, Clostridium ramosum*, and *Coprobacillus* positively correlated with the severity of the disease[33]. A study in a Chinese population reported that intestinal infection by SARS-CoV-2 can induce the production of proinflammatory factors such as IL-18. IL-18 is a proinflammatory cytokine produced by multiple enteric cells, including intestinal epithelial cells, immune cells, and the enteric nervous system, is shown increased in the serum of COVID-19 patients. IL-18 levels seem to correlate with an abundance of *Peptostreptococcus*, *Fusobacterium*, and *Citrobacter*, indicating changes in gut microbiota[34].

Obesity presents changes in microbiota, dysregulation of cytokine profiles, and higher levels of ACE2 in adipocytes[35]. As the opposite, an adequate fiber intake and whole grains diet improves intestinal microbiome composition, reduces intestinal inflammation markers like CRP, IL-6, and TNF-α[36]. Besides the colon and intestines, the liver is another organ the SARS-CoV-2 could affect[29].

LIVER INJURY IN COVID-19 PATIENTS

The few reports regarding liver damage by COVID-19 come from autopsies carried out in different hospital centers. The incidence of liver damage in patients with COVID 19 ranges from 14% to 53%[37]. Patients with elevated liver function tests were more likely to have a moderate-high degree fever, and these elevations were significantly more prevalent in male patients (68.67% *vs* 38.36%). It is important to mention that it is difficult to define how COVID-19 generates liver damage since patients at the time of hospitalization usually have chronic diseases such as non-alcoholic hepatic steatosis, which increases the progression of the disease, hepatitis C such as those reported by Schmit[20,38]. Among the biochemical indicators, there have been reports of elevated aminotransferases approximately on the tenth day of hospitalization[39].

Patient biopsies reveal the presence of hepatocyte mitosis with acidophilic bodies, moderate inflammation, and ballon degeneration. In the SARS virus epidemic in 2003, a study reported the elevation of aminotransferases in a range of 300-400, and prominent mitoses, which refers to what researchers have published in various studies

from these pandemics. The authors assumed that the prominent mitosis was likely due to a hyperproliferative state and cell cycle arrest[40].

Some researchers hypothesized that the direct action of the virus on liver cells causes centrilobular, periportal necrosis without significant inflammation compatible with acute liver damage. Also, the authors report that development of cholestasis and a great reactive biliary proliferation as consequences of the virus are to be expected. They further consider the possibility that the virus enters the liver through the portal vein[38]. The definitive mechanism by which liver injury occurs in COVID-19 patients remains unclear. There are multiple theories of the pathophysiology of the viral infection that could explain this phenomenon: (1) ACE2-mediated direct viral infection of hepatocytes; (2) Critically-ill status and immune-mediated injury; or (3) Drug hepatotoxicity[11] (Figure 2).

Liver damage by COVID-19 can be clarified thanks to the severe inflammatory response and cytotoxicity of the active replication with ACE2 receptors expressed in the liver, especially in cholangiocytes and epithelial cells of the bile duct, which is why the liver is also considered a target organ for SARS-CoV2 infection[41].

Fiel *et al*[42] found this elevation associated with pharmacological treatment with lopinavir/ritonavir. However, a review demonstrated that aminotransferases are only significantly elevated in severe COVID-19 cases. Drug toxicity has served as one mechanism for COVID-19-associated liver injury, damage that is secondary and does not make them susceptible to viral infection. However, little is known about the incidence of hepatotoxicity of various drugs used in COVID-19. Understandably, efforts are currently made regarding this concern. These efforts will prove important in developing a reasonable intervention and reducing the harmful effects of drug-induced hepatotoxicity for patients[29].

SARS-COV-2 AND THE PANCREAS

Expressed in the pancreas is the angiotensin converting enzyme 2 specifically in the exocrine glands, and islets[43] therefore, it is susceptible to SARS-CoV-2 infection. In a cohort of 121 patients with COVID-19 in China, 10% had increased lipase levels but only 4% showed pancreas enlargement or dilatation in computerized tomography (CT) scans[44].

In another cohort of 71 patients in the United States, 12% had increased lipase levels but only 3% exceeded three times the upper normal limit. None of the patients had abnormal pancreas images in CT scans[45]. In a cohort of 83 patients with COVID-19 in the United States, 16.8% had increased lipase levels (three times the upper limit). Researchers have associated high lipase levels with admission to the intensive care unit and intubation after a multivariable-adjusted model[46]. In a retrospective pooled analysis, the pooled prevalence of hyperlipasemia was 12% and the pooled odds ratio for severe COVID-19 was 3.143[47]. The ACE2 receptor is also highly expressed in pancreatic islet cells[43]; therefore, SARS-CoV-2 infection can theoretically cause islet damage resulting in acute diabetes, which associates to patients with pancreatic injury and high blood sugar. Mechanisms by which pancreatic injury could occur include the direct cytopathic effects of SARS-CoV-2 or indirect systemic inflammatory and immune-mediated cell responses, resulting in organ damage or secondary enzyme abnormalities. Antipyretics, which most of the patients in this study took before admission, could also cause drug-related pancreatic injury[48]. However, more information to understand the role of pancreatic injury in patients with COVID-19 is needed.

TREATMENTS OF GI SYMPTOMS

At the current stage of the COVID-19 pandemic, several vaccines are in their last stages of authorization for emergency use[49,50]. While full distribution will continue as a challenge, hopes of major population immunity are coming close. Yet, until we have a more resistant population, respiratory complications will continue as the major symptom reported during a COVID-19 infection. Interestingly, other lesser-known indicators that manifest, such as those of the GI which include vomiting, nausea, and diarrhea[12,51]. Many studies have shown that vomiting and nausea can be present in upwards of 30% and 15% of patients[12]. Interestingly enough, in a pediatric setting, one reported case showed patients with no-respiratory affliction who were all COVID-19 positive; all presented GI alterations. Several showed gastroenteritis, another

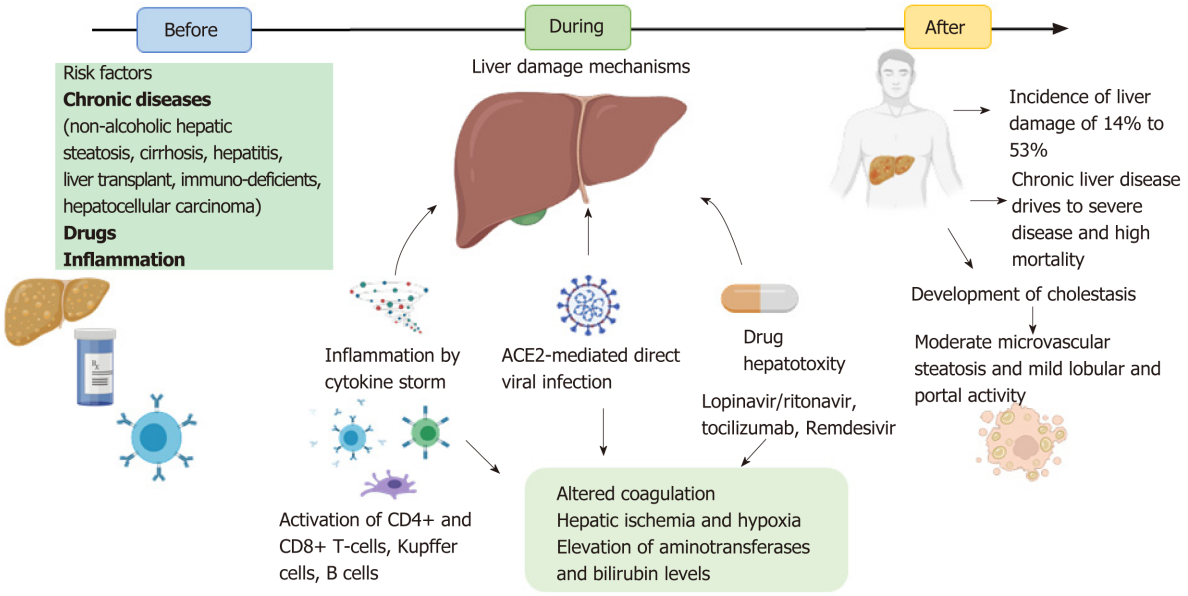


Figure 2 Proposed process of liver damage. Before severe acute respiratory syndrome-coronavirus-2 infection, there are risk factors considered that could be poor prognostic factors, such as chronic diseases, the use of drugs that affect the liver and the inflammation process. The virus can infect the liver through the portal vein. There are three proposed mechanisms of liver damage: inflammation induced by cytokine storm and activation of hepatic immunity, angiotensin-converting enzyme 2-mediated direct viral infection of hepatocytes, epithelial cells, and cholangiocytes, and drug hepatotoxicity mediated by some antivirals employed for coronavirus disease-2019 (COVID-19) treatment. The three mechanisms culminate in altered coagulation, hepatic ischemia, and elevation of aminotransferases and bilirubin levels. Following this, the incidence of liver damage derived from COVID-19 is up to 53%, which could develop cholestasis and reach high mortality risk. ACE2: Angiotensin-converting enzyme 2.

patient appendicitis, and yet another, hydronephrosis[52]. As more data becomes available, GI manifestations such as loss of appetite seem to be direct signs of COVID-19. Counter to the GI manifestations brought about by COVID-19, several drugs used to combat the effects of the virus have secondary side effects. Drugs like remdesivir, hydroxychloroquine, favipiravir, ivermectin, and azithromycin can induce side effects such as vomiting, nausea, elevated liver enzymes, weight loss, abdominal pain, and others[53]. The Table 1 display wide information.

In addition, COVID-19 patients can present a hyper-inflammatory state, with systemic response and cytokine storm mediated by IL-6, IL-8, and TNF-α, which can induce platelet activation and thrombosis, also presenting endothelial dysfunction due to direct virus damage and inflammation[54]. Heparin is used in COVID-19 patients as prophylactic therapy to prevent thrombosis. However, heparin-induced-thrombocytopenia (HIT) after administering low doses may not be enough to counteract the hypercoagulable state, leading to coagulation problems in these patients[55]. Heparin treatment, by a direct interaction between heparin and platelets, induces platelet clumping or sequestration. This event occurs within the first 48-72 h after starting treatment and generates mild and transient thrombocytopenia[56]. In some cases, thrombosis could be associated with HIT after heparin cessation[57].

In the GI system, the intestinal microbiota plays a crucial role in the correct balance and maintenance. If unbalanced component processing becomes inefficient, direct damage to the intestinal mucosa results in more accessible routes for viral infection[12,58]. Studies have confirmed that probiotics can assist in this treatment; both bifidobacterium and lactic acid can help induce antibody production[12]. It is important to mention that patients with severe GI symptoms require a nutritional risk assessment, as it becomes a predictor of outcome both in the long term and the short term[59].

COVID-19 individuals presenting irritable bowel disease are of particular interest since this condition warrants the use of immunosuppressants and steroids. Interestingly, vedolizumab and ustekinumab do not increase the risk of COVID-19, hence patients can continue its use safely. Yet, thiopurines, anti-tumor necrosis factor (anti-TNF) agents, and JAK inhibitors may continue to present a risk. In mild cases, 5-ASA and budesonide use are reasonable[53]. We should take special consideration to outweigh the benefit against the risk for each case. Also, unless emergent, patients should defer all surgical procedures until pandemic conditions rescind[60]. We should

Table 1 Side effects of most common drugs during coronavirus disease-2019 treatment

Pharmacological intervention	Mechanism of action	Adverse effects	Ref.
Hydroxychloroquine	Elevated endosomal pH; Disruption of lysosome-endosome fusion. Inhibition of cell-virus fusion when interacting with N-terminal domain of the SARS-CoV-2 peak	Q-T segment prolongation; Gastrointestinal Adverse Effects	[62-64]
Chloroquine	Inhibits RNA-dependent polymerases, decreases endosomal iron release required for DNA replication, and inhibits glycosylation of viral envelope glycoproteins	Gastrointestinal adverse effects; visual and extrapyramidal disturbances; Arrhythmogenic cardiotoxicity	[65-67]
Remdesivir	Transcription Inhibitor	Caution in patients with severe renal impairment [estimated glomerular filtration rate (eGFR) < 30 mL/min/1.73] or severe liver disease	[68]
Lopinavir/Ritonavir	Lopinavir binds to the viral protease and prevents the cleavage of the Gag-Pol polyprotein, resulting in the production of non-infectious immature viral particles. Ritonavir increases the plasma concentration of lopinavir by inhibiting the metabolism of cytochrome P450 3A (CYP3A)	Gastrointestinal adverse effects	[64, 65,68-72]
Ribavirin	Interferes with RNA polymerase and viral protein synthesis	Hemolytic anemia; Leukopenia; Teratogenic	[68]
Interferon	Degradation of viral RNA; Alteration of RNA transcription; Inhibition of protein synthesis and apoptosis	Worsening psychiatric conditions, cytopenia, and uncontrolled seizures	[68]
Cortocosteroids, dexametasone	Inhibitor of the inflammatory process	Impair the immune response; Bacterial pneumonia risk; Hyperglycemia; Osteoporosis; Hypertension	[68-70]
Azithromycin	Bacteriostatic antibiotics; Anti-inflammatory effects Immunomodulatory effects	QTc with the risk of arrhythmias	[71, 73]
Heparin	Antiplatelet	Risk GI symptoms; Bleeding; Heparin-induced thrombocytopenia	[74]
Favipiravir	Competitive inhibitor of RNA-dependent RNA polymerase	GI adverse effects; liver injury	[75, 76]

SARS-CoV-2: Severe acute respiratory syndrome-coronavirus-2; GI: Gastrointestinal.

consider patients with Crohn's disease for colectomy with end ileostomy. An important aspect to take into consideration is comorbidities, such as diabetes and hypertension, which are exacerbators of damage in COVID-19. As expected, comorbidities become paramount in symptom management, because of the high risk they represent[60,61].

CONCLUSION

By now, we know that GI symptoms in COVID-19 disease such as diarrhea are related to gut microbiota alterations that alter profile cytokines, either by SARS-Cov-2 ACE2 alterations or as a secondary effect of antibiotic and antiviral drugs employed in treatment. However, additional research is needed for the hepatic and pancreatic manifestations that aggravates the patient´s situation, and a deeper understanding of the sequelae after symptoms of the disease. Until now, the knowledge that we have mainly involves the host; however, we must not ignore the pathogenicity of the virus and the recent variants that are currently circulating since these could in the future serve to explain in greater detail the mechanisms involved in the intestinal damage or with the presentation of GI symptoms that can accompany COVID-19 respiratory disease.

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MINIREVIEWS

Adult pancreatoblastoma: Current concepts in pathology

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Abstract

Adult pancreatoblastoma is an exceptionally rare malignant tumour of the pancreas that mimics other solid cellular neoplasms of the pancreas, which may pose diagnostic difficulties. Because of its rarity, little is known about its clinical and pathologic features. This article reviews the clinical and pathologic features of pancreatoblastoma in adults including differential diagnosis, treatment, and follow-up. Although pancreatoblastoma commonly occurs in childhood, there have now been more than 70 adult pancreatoblastomas described in the literature. There is a slight male predominance. There are no symptoms unique to pancreatoblastomas and adult patients are frequently symptomatic. The most common presenting symptom is abdominal pain. Grossly, the tumours are often large and well-circumscribed. Microscopically, pancreatoblastomas are composed of neoplastic cells with predominantly acinar differentiation and characteristic squamoid nests. These tumours are positive for trypsin, chymotrypsin, lipase, and BCL10. Loss of heterozygosity on chromosome 11p is the most common molecular alteration in pancreatoblastomas. Adult pancreatoblastomas are aggressive tumours with frequent local invasion, recurrence, and distant metastasis. Treatment consists of surgical resection. Chemotherapy and radiotherapy may have a role in the treatment of recurrent, residual, unresectable, and metastatic disease. It is important to distinguish pancreatoblastomas from morphological mimics such as acinar cell carcinomas, solid pseudopapillary neoplasms, and pancreatic neuroendocrine neoplasms.

Key Words: Pancreas; Adult pancreatoblastoma; Pancreatic cancer; Solid pancreatic mass; Non-ductal pancreatic tumours

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Core Tip: Adult pancreatoblastomas are extremely rare tumours of the pancreas. They are composed of neoplastic cells with multiple lines of differentiation and characteristic

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squamoid nests. They mimic other neoplasms of the pancreas, which may give rise to diagnostic difficulties. This article provides an up-to-date review of the clinical and pathologic features of pancreatoblastoma in adults, including differential diagnosis, treatment, and follow-up.

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INTRODUCTION

Pancreatoblastoma is a malignant epithelial neoplasm of the pancreas composed of cells with predominantly acinar differentiation and characteristic squamoid nests. Neuroendocrine, ductal and less commonly, mesenchymal differentiation can be seen but are often less extensive[1-3]. Less than 1% of pancreatic neoplasms are pancreatoblastomas[4,5]. Pancreatoblastoma commonly occurs in childhood, accounting for 25% of pancreatic neoplasms occurring in the first decade of life, with a mean age of approximately 4 years[1].

Adult pancreatoblastoma is extremely rare. Hence, little is known about its clinical and pathologic features. Furthermore, pre-operative diagnosis can be quite challenging because of the considerable overlap with other cellular neoplasms of the pancreas.

This article provides an up-to-date review of the clinical and pathologic features of pancreatoblastoma in adults, including cytology, molecular pathology, differential diagnosis, treatment, and follow-up.

EPIDEMIOLOGY

Adult pancreatoblastomas are exceptionally rare. To date, only 74 adult pancreatoblastomas have been reported in the literature, mostly in the form of isolated case reports and small series[6-10]. The mean age at diagnosis is 41 years (range, 18-78 years). There is a slight male predilection, with a male-to-female ratio of 1.2:1.

AETIOLOGY

The aetiology is unknown. Although most tumours are sporadic[11-15], few adult pancreatoblastomas have been described in the setting of familial adenomatous polyposis (FAP)[8,9]. Rare cases in children have been associated with Beckwith-Wiedemann syndrome[16,17].

CLINICAL PRESENTATION

Most patients are symptomatic, with very few cases discovered incidentally during routine examination and imaging[4,10,11]. There are no symptoms unique to pancreatoblastomas. The most common presenting symptom is abdominal pain[2]. Other clinical features include abdominal mass, weight loss, nausea, jaundice, and diarrhoea [6,14,15,18,19]. Rarely, patients may present with upper gastrointestinal bleeding[12].

Most adult pancreatoblastomas arise in the head of the pancreas. Of the 74 adult pancreatoblastomas described in the literature, localization data were available in 69 cases. The head of the pancreas was involved in 52.1% of cases (36 patients); the tail in 30.4% of cases (21 patients); the body in 14.5% of cases (10 patients); the body and tail in 1.5% of cases (1 patient); and the ampulla of vater in 1.5% of cases (1 patient).

Elevated serum levels of CA19-9[14,15] as well as corticotropin releasing hormone secretion[20] rarely occurs in adult pancreatoblastomas. Serum alpha fetoprotein

(AFP) is elevated in some pancreatoblastomas[4,7,13,21,22]. In addition, AFP may be detected immunohistochemically in tumours associated with elevated serum levels of AFP[3,4,22]. Serum AFP is frequently elevated in children[4,10,13] with levels often in excess of 1000 μg/L[3]. In contrast, AFP is not consistently elevated in adults[2,7,10,13]. When present, elevated serum AFP has been used as a marker of tumour recurrence or disease progression because AFP levels should decrease or normalize with successful treatment[7,13,21].

It is important to note that elevated AFP is not specific for pancreatoblastoma in a patient with a pancreatic mass. Pancreatic ductal adenocarcinomas[23] and pancreatic acinar cell carcinomas[24,25] have been associated with elevated serum AFP. Furthermore, AFP is widely used as a tumour marker for hepatocellular carcinoma (HCC). However, the limitations of AFP in detecting HCC includes the poor sensitivity in detecting small tumours and elevated levels of AFP in patients with chronic liver disease without HCC. To overcome this limitation, the *Lens culinaris* agglutinin-reactive AFP (AFP-L3) has been found to be highly specific and useful not only for early detection of HCC but also for predicting the risk of development of HCC in patients with chronic liver disease[26,27]. However, AFP-L3 or other isoforms of AFP are yet to be extensively studied in pancreatoblastomas.

Malignant behaviour is prominent in adult pancreatoblastomas. Approximately 59% of adult patients with pancreatoblastoma develop metastases at the time of diagnosis or afterwards in the course of the disease. The liver is the most common site of metastasis[2,12,15,28] followed by lymph nodes[4,5,29], and lung[4,5,7,15]. Chest wall[5], breast[15], bone[30], and brain metastases[31] are extremely rare. Tumours can invade adjacent structures such as the duodenum, spleen, common bile duct, portal vein, and superior mesenteric vessels[2,4,7,19,29].

IMAGING

There are no significant differences in the imaging findings of adult and paediatric patients[2,32]. Pancreatoblastomas are large well-defined heterogenous masses with low to intermediate signal intensity on T1-weighted images and high signal intensity on T2-weighted images. Enhancement is a common feature on contrast-enhanced computed tomography images and may be present on magnetic resonance imaging. Calcifications when present may be rim-like or clustered[19,33]. On ultrasound, pancreatoblastomas are well-demarcated solid masses inseparable from the pancreas with mixed echogenicity[33].

CYTOLOGY

Fine needle aspiration specimens are composed of cellular singly dispersed and/or clustered polygonal cells. The cells have round to oval nuclei with fine chromatin pattern, small indistinct nucleoli, and moderate amounts of amphophilic or eosinophilic cytoplasm[3,9]. Squamoid nests or corpuscles are best appreciated in cell block preparations. They are composed of plump epithelioid cells with abundant cytoplasm [7,9].

PATHOLOGY

Grossly, the tumours are solitary, solid, well-circumscribed, and often encapsulated. Pancreatoblastomas are usually large, averaging 8 cm in diameter (range, 1.8–30 cm)[2,3,6]. On cut section, the tumours have yellow to tan fleshy lobules separated by dense fibrous bands. Foci of haemorrhage and necrosis may be present. Rarely, pancreatoblastomas may undergo cystic change or show gross extension into the adjacent peripancreatic soft tissue[4].

Microscopically, pancreatoblastomas are composed of cellular well-delineated lobules separated by dense fibrous bands, often imparting a geographic low power appearance (Figure 1A). The dense fibrous bands between the lobules are composed of spindled cells with varying amounts of collagen (Figure 1B). Tumours predominantly show acinar differentiation; however, ductal, neuroendocrine and less commonly, mesenchymal differentiation may be present[1-3]. Solid areas with sheets of cells often alternate with areas with acinar differentiation. The acinar units comprise small cells

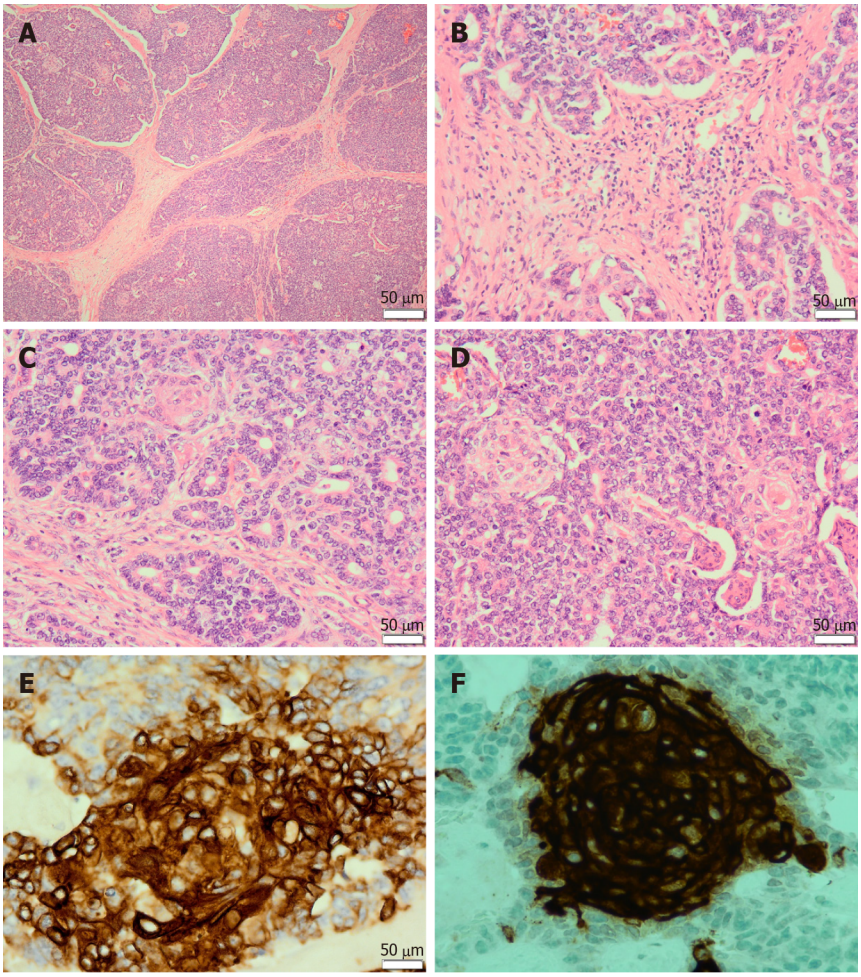


Figure 1 Pancreatoblastoma. A: The tumour is composed of lobules separated by dense fibrous bands, imparting a geographic low power appearance [Haematoxylin and Eosin (H&E) staining, 40 ×]; B: The dense fibrous bands between the lobules are composed of spindled cells with varying amounts of collagen (H&E staining, 200 ×); C: The tumour predominantly shows acinar differentiation. The acinar units are composed of neoplastic cells arranged around central lumina (H&E staining, 200 ×); D: The tumour shows characteristic squamoid nests. Squamoid nests are large islands of plump epithelioid cells with abundant eosinophilic cytoplasm (H&E staining, 200 ×); E: The squamoid nests are immunoreactive for AE1/AE3 (400 ×); F: The tumour shows immunolabeling for CD10 limited to the squamoid nests (400 ×).

with granular cytoplasm arranged around central lumina (Figure 1C). The cells have round to oval nuclei with single prominent nucleoli[1,3,4].

The defining histological feature of pancreatoblastoma is the squamoid nests. Squamoid nests vary from large islands of plump epithelioid cells to whorled nests of spindled cells showing mild to frank keratinization. The cells of the squamoid nests are often distinct from surrounding acinar cells. They are larger than surrounding cells with abundant eosinophilic to clear cytoplasm and without cytological atypia (Figure 1D). The amount of squamoid nests can vary both within and between tumours.

Pancreatoblastomas typically express trypsin, chymotrypsin, lipase, and BCL10. The granules are periodic acid-Schiff (PAS)-positive and resistant to diastase (PASD)[3,4]. Focal expression of chromogranin A and synaptophysin may be present. Squamoid nests may be positive for EMA, AE1/AE3 (Figure 1E) or CD10 (Figure 1F). In addition, patchy nuclear and cytoplasmic expression of β-catenin may be seen.

The staging of pancreatoblastoma follows the TNM classification of carcinoma of the exocrine pancreas[3].

MOLECULAR PATHOLOGY

Loss of heterozygosity on chromosome 11p is the most common molecular alteration in pancreatoblastomas, occurring in 86% of cases. Molecular alterations in the adenomatous polyposis coli (APC)/β-catenin signalling pathway have also been

identified in 67% of pancreatoblastomas, including biallelic inactivation of the *APC* gene and activating mutations of CTNNB1 (β-catenin) gene. Biallelic inactivation of the *APC* gene has been identified in a patient with pancreatoblastoma arising in the setting of FAP[8]. Interestingly, aberrations in the APC/β-catenin pathway have been implicated in the development of hepatoblastoma, a tumour associated with Beckwith-Wiedemann syndrome[8,34].

Recent RNA sequencing studies have identified molecular aberrations in the fibroblast growth factor receptor (FGFR) signalling pathway. These include somatic FGFR1 mutation, *FGFR2* gene rearrangement, and a high mRNA expression of fibroblast growth factor (FGF) receptors 1, 3 and 4 as well as of their ligands, FGF3 and FGF4[18].

The most frequent recurrent molecular alterations identified in pancreatic ductal adenocarcinomas, including mutations in *KRAS*, *TP53*, and *CDKN2A/p16* genes, are typically lacking in pancreatoblastomas, suggesting that pancreatoblastomas are genetically distinct from pancreatic ductal adenocarcinomas[8]. Loss of SMAD4/DPC4 expression is rare in pancreatoblastomas[8,35].

DIFFERENTIAL DIAGNOSIS

Pancreatoblastomas are distinct from the more common pancreatic ductal adenocarcinoma, and it is generally easy to differentiate them on the basis of morphology. The differential diagnosis of pancreatoblastoma includes solid cellular neoplasms of the pancreas such as acinar cell carcinomas, solid pseudopapillary neoplasms, and pancreatic neuroendocrine neoplasms (PanNENs).

There are a number of clinical and morphological similarities between acinar cell carcinomas and pancreatoblastomas. Acinar cell carcinomas are rare, accounting for 1%-2% of pancreatic neoplasms in adults and about 15% in children[1]. Acinar cell carcinomas have a poor prognosis, with a mean survival of 18-24 mo and a 3-year survival rate of 26%[36,37]. Both acinar cell carcinomas and pancreatoblastomas present with non-specific clinical symptoms such as abdominal pain, abdominal mass, and weight loss. In addition, both tumours are cellular neoplasms with acinar differentiation. Neoplastic cells are often polarized around central lumina. The cells contain PASD-positive cytoplasmic granules. Furthermore, acinar cell carcinomas and pancreatoblastomas are typically immunoreactive for trypsin, chymotrypsin, lipase, and BCL10. However, the distinguishing feature is the characteristic squamoid nests seen in pancreatoblastomas.

Solid pseudopapillary neoplasm of the pancreas is a low-grade malignant neoplasm characterized by cells with solid and pseudopapillary growth patterns. Approximately 1%-2% of pancreatic neoplasms are solid pseudopapillary neoplasms, and they frequently occur in girls and young women[3]. Microscopically, solid pseudopapillary neoplasms are composed of poorly cohesive monomorphic epithelial cells arranged around hyalinized fibrovascular stalks, forming solid and pseudopapillary structures. The nuclei frequently show indentations, clefts, and grooves. Typically, these tumours contain scattered PASD-positive hyaline globules, foamy histiocytes, cholesterol clefts, and foreign body giant cells[1]. Solid pseudopapillary neoplasms are positive for nuclear and/or cytoplasmic β-catenin, CD56, CD10, vimentin, and cyclin D1. Unlike pancreatoblastomas, the prognosis of solid pseudopapillary neoplasm of the pancreas is excellent.

PanNENs constitute about 2%-5% of pancreatic neoplasms[3]. They are architecturally diverse and can be confused with pancreatoblastomas. In addition, pancreatoblastomas can focally express neuroendocrine markers. In contrast, pancreatic neuroendocrine tumours are composed of cells with amphophilic to eosinophilic cytoplasm and the nuclei have characteristic salt and pepper chromatin. Typically, pancreatic neuroendocrine tumours strongly express synaptophysin, chromogranin A, and CD56. Features that favour a diagnosis of pancreatoblastoma include predominant acinar differentiation, squamoid nests, PASD-positive cytoplasmic granules, and expression of trypsin, chymotrypsin, lipase, and BCL10.

OUTCOME

There are no established treatment guidelines for pancreatoblastoma. Treatment consists of surgical resection with a variable combination of chemotherapy, radiotherapy, or targeted therapy (Table 1).

Table 1 Treatment and outcome of adult pancreatoblastoma

Ref.	Treatment	Follow-up (mo)	Outcome
Charlton-Ouw *et al*[39], 2008	Surgical resection, chemotherapy, RT	60	NED
Levey and Banner[40], 1996	Surgical resection	4	DOD
Palosaari *et al*[29], 1986	Surgical resection, chemotherapy, RT	15	AWD
Rajpal *et al*[13], 2006	Surgical resection, chemotherapy	17	DOD
Dunn and Longnecker[41], 1995	Surgical resection, chemotherapy	11	DFUD
Zhu *et al*[42], 2005	Chemotherapy	9	AWD
Du *et al*[14], 2003	Surgical resection	6	NED
Hoorens *et al*[43], 1994	Surgical resection	30	NED
Robin *et al*[44], 1997	Surgical resection, chemotherapy	7	DOD
Gruppioni *et al*[45], 2002	Surgical resection	10	NED
Benoist *et al*[12], 2001	Surgical resection, chemotherapy	36	NED
Mumme *et al*[46], 2001	Surgical resection, chemotherapy, RT	9	DOD
Salman *et al*[5], 2013	Surgical resection	30	NED
Salman *et al*[5], 2013	Surgical resection, chemotherapy, RT	41	NED
Salman *et al*[5], 2013	Surgical resection, chemotherapy, ablation of liver mets	51	DOD
Hayasaki *et al*[47], 1999	Surgical resection	15	NED
Sheng *et al*[48], 2005	Surgical resection, chemotherapy, RT, TACE	26	DOD
Balasundaram *et al*[15], 2012	Chemotherapy	1	DFUD
Klimstra *et al*[4], 1995	Surgical resection	5	NED
Klimstra *et al*[4], 1995	None	5	DOD
Klimstra *et al*[4], 1995	Surgical resection	10	DOD
Klimstra *et al*[4], 1995	Surgical resection	15	NED
Klimstra *et al*[4], 1995	Chemotherapy, RT	38	DOD
Rosebrook *et al*[32], 2005	Surgical resection	NA	NA
Montemarano *et al*[19], 2000	Surgical resection	NA	NA
Abraham *et al*[8], 2001	NA	NA	NA
Abraham *et al*[8], 2001	NA	NA	NA
Boix *et al*[20], 2010	Surgical resection	3	DOD
Pitman and Faquin[7], 2004	Surgical resection, chemotherapy, RT	108	AWD
Savastano *et al*[49], 2009	Surgical resection, chemotherapy, RT	NA	NED
Cavallini *et al*[10], 2009	Surgical resection	51	NED
Cavallini *et al*[10], 2009	Surgical resection	15	NED
Hammer and Owens[28], 2013	Surgical resection	NA	NA
Zhang *et al*[50], 2015	Surgical resection, chemotherapy	NA	NED
Ohike *et al*[51], 2008	Surgical resection	108	NED
Chen *et al*[52], 2018	Hepatic transarterial chemoembolization (TACE)	48	DOD
Yamaguchi *et al*[53], 2018	Surgical resection, chemotherapy	13	DOD
Nunes *et al*[54], 2018	Palliative care	3	DOD
Vilaverde *et al*[55], 2016	Surgical resection, chemotherapy	12	DOD
Zouros *et al*[30], 2015	Surgical resection, chemotherapy, RT	13	DOD
Kuxhaus *et al*[56], 2005	NA	NA	NA

Comper *et al*[57], 2009	Surgical resection	NA	NA
Comper *et al*[57], 2009	Surgical resection	NA	NA
Gringeri *et al*[58], 2012	Surgical resection, chemotherapy, stereotactic RT	44	NED
Redelman *et al*[59], 2014	Surgical resection	NA	NA
Tabusso *et al*[6], 2017	Surgical resection, chemotherapy, RT	10	AWD
Tabusso *et al*[6], 2017	Surgical resection	15	NED
Liu *et al*[60], 2020	Surgical resection	24	NED
Reid *et al*[9], 2019	NA	72.2	DOD
Reid *et al*[9], 2019	NA	17.9	AWD
Reid *et al*[9], 2019	NA	3.6	DOD
Reid *et al*[9], 2019	NA	85	DOD
Reid *et al*[9], 2019	NA	143.7	DOD
Reid *et al*[9], 2019	NA	13.6	NED
Reid *et al*[9], 2019	NA	0.8	DOD
Reid *et al*[9], 2019	NA	6.5	NED
Reid *et al*[9], 2019	NA	348	AWD
Reid *et al*[9], 2019	NA	88	AWD
Reid *et al*[9], 2019	NA	91	AWD
Terino *et al*[61], 2018	Chemotherapy	NA	NA
Morrissey et *al*[62], 2020	Surgical resection, chemotherapy	2	NED
Berger *et al*[8], 2020	Surgical resection, chemotherapy	18	DOD
Berger *et al*[8], 2020	Surgical resection, chemotherapy, RT, splenectomy	24	DOD
Berger *et al*[8], 2020	Surgical resection, chemotherapy, immunotherapy, RT	17	DOD
Berger *et al*[8], 2020	Chemotherapy, tyrosine kinase inhibitor therapy	15	DOD
Zhang *et al*[11], 2020	NA	NA	NA
Zhang *et al*[11], 2020	NA	NA	NA
Zhang *et al*[11], 2020	NA	NA	NA
Zhang *et al*[11], 2020	NA	NA	NA
Zhang *et al*[11], 2020	NA	NA	NA
Zhang *et al*[11], 2020	NA	NA	NA
Zhang *et al*[11], 2020	NA	NA	NA
Elghawy *et al*[31], 2021	Chemotherapy, autologous hematopoetic cell transplantation	57	AWD
Snyder *et al*[63], 2020	Surgical resection, chemotherapy, GKRS	63	NED

RT: Radiotherapy; GKRS: Gamma knife radiosurgery; DOD: Died of disease; AWD: Alive with disease; DFUD: Died from unrelated disease; NED: No evidence of disease; NA: Not available.

Of the 74 cases of adult pancreatoblastomas described in the literature, outcome data were available in 57 cases. The mean follow-up time was 36 mo (range, 0.8-348 mo). Forty-two percent (24 cases) of patients died of the disease at a mean interval of 27 mo (range, 0.8-143.7 mo); 4% (2 cases) of patients died from unrelated causes (cerebral haemorrhage and pulmonary artery embolus); 16% (9 cases) of patients were alive with disease; and 38% (22 cases) of patients had no evidence of disease (Table 1).

Although long-term survival has been observed in some adults, the prognosis of pancreatoblastoma in children may be more favourable than in adults[1,4,13,14]. Poor prognostic factors include the presence of metastases and unresectable disease[3]. Chemotherapy and radiotherapy may have a role in the treatment of recurrent, residual, unresectable and metastatic disease[3,38]. Because of the tendency for

recurrence and metastasis, long-term follow-up is advised for these patients[38].

CONCLUSION

In summary, adult pancreatoblastomas are extremely rare. Although these tumours typically occur in children, pancreatoblastomas should be considered in the differential diagnosis of solid pancreatic tumours in adults. An appreciation of distinctive squamoid nests, predominant acinar differentiation, and expression of trypsin, chymotrypsin, lipase, and BCL10 are important for the accurate diagnosis of pancreatoblastomas. These tumours are aggressive with frequent local invasion, recurrence, and distant metastasis. They must be distinguished from morphological mimics. There is a need for further research to better understand the molecular drivers of pancreatoblastomas, identify druggable molecular targets, and, most importantly, improve patient care.

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MINIREVIEWS

Prevention of vertical transmission of hepatitis B virus infection

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Abstract

Hepatitis B virus (HBV) is the leading cause of chronic viral hepatitis. Annually, almost two million children younger than 5 years acquire the infection, mostly through vertical or horizontal transmission in early life. Vertical transmission of HBV is a high efficacy phenomenon ranging, in the absence of any preventive interventions, from 70% to 90% for hepatitis e antigen positive mothers and from 10% to 40% for hepatitis e antigen-negative mothers. Maternal viraemia is a preeminent risk factor for vertical transmission of HBV. Maternal screening is the first step to prevent vertical transmission of HBV. Hepatitis B passive and active immunoprophylaxis at birth together with antiviral treatment of highly viraemic mothers are the key strategies for global elimination of HBV infection. Strategies are needed to promote implementation of birth-dose vaccination and hepatitis B immunoglobulins in low- and middle-income countries where the prevalence of the infection is at the highest.

Key Words: Hepatitis B; Vertical transmission; Hepatitis B vaccine; Hepatitis B immune globulin; Neonatal immunoprophylaxis; Tenofovir alafenamide fumarate

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Core Tip: Hepatitis B is one of the main causes of morbidity and mortality worldwide. Vertical transmission is the main transmission route, especially in areas with high prevalence of the infection. Maternal viraemia is a preeminent risk factor for vertical transmission of hepatitis B virus (HBV). Breastfeeding is recommended, although all the conditions leading to maternal-foetal microtransfusions with HBV-infected

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maternal blood increase the risk of vertical transmission. Neonatal immunoprophylaxis at birth represent the most important approach to prevent HBV infection. The aim of the present narrative review is to summarise the knowledge on prevention of vertical transmission of HBV infection.

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INTRODUCTION

Hepatitis B virus (HBV) is the leading cause of chronic viral hepatitis and a major cause of acute and chronic liver disease and associated morbidity and mortality worldwide[1]. According to the latest estimation, in 2016 there were 291 million people chronically infected with HBV in the world corresponding to a global prevalence of 3.9%. Annually, almost two million children younger than 5 years acquire the infection. The highest prevalence has been reported in Africa and in the Western Pacific area. In these regions the coverage with the birth vaccination dose is at the lowest, mostly through vertical transmission in early life[1]. Vertical transmission or infections acquired during early infancy are still responsible for most chronic HBV infections in adults, especially in the areas with high prevalence of the infection[2,3]. Hepatitis B passive and active immunoprophylaxis at birth together with antiviral treatment of highly viraemic mothers are the key strategies for global of HBV infection [4]. According to latest World Health Organization (WHO) estimates, the relative amount of children under 5 years of age chronically infected with HBV dropped to under 1% in 2019, down from around 5% in the pre-vaccine era[5]. In 2019, coverage of three doses of the vaccine reached 85% worldwide compared to around 30% in 2000. However, coverage of the hepatitis B vaccine birth dose remains uneven. Global coverage of the HBV birth dose is 43%, while coverage in the WHO African Region is only 6%[5].

Breast-feeding does not entail any additional risk of transmission in infants who receive a correct immunoprophylaxis[6]. The aim of the present narrative review is to summarise the knowledge on prevention of vertical transmission of HBV infection.

VERTICAL TRANSMISSION OF HBV: DEFINITION, TIMING AND TRANSMISSION RATE

Vertical transmission of HBV is defined as transmission occurring during pregnancy and in the perinatal period from the HBV-infected mother to the foetus or to the child, resulting in positivity at 6-12 mo of life of the hepatitis B surface antigen (HBsAg) or HBV DNA in infants[7]. Overall, vertical transmission of HBV is a high efficacy phenomenon ranging, in the absence of any preventive interventions, from 70% to 90% for hepatitis e antigen (HBeAg) positive mothers and from 10% to 40% for HBeAg-negative mothers. The high success rate of immunoprophylaxis provided to newborns in reducing the incidence of HBV transmission suggests that most vertical transmissions occur at or near the time of birth. Intrauterine infections take place in < 15% of pregnancies.

RISK FACTORS FOR VERTICAL TRANSMISSION OF HBV

Maternal viraemia, identified through the detection of HBV DNA or through the positivity of its surrogate markers HBsAg and HBeAg, is a preeminent risk factor for vertical transmission of HBV. HBeAg-positive mothers and mothers with high circulating concentrations of HBV DNA (> 10^6 IU/mL) have the highest risk of transmission[8,9]. All the conditions leading to maternal-foetal microtransfusions with HBV-infected maternal blood increase the risk of vertical transmission. Microtrans-

fusions could occur intrauterine, during labour, or at delivery. Placental leakages due to threatened preterm delivery or abortion, amniocentesis or chorionic villus sampling, and prolonged uterine contractions could be associated with maternal microtransfusions. The exposure of the neonate to the maternal HBV-infected cervical secretions and blood is possible during labour and delivery.

Mode of delivery

The mode of delivery has been examined as a potential risk factor for vertical transmission of HBV, but the resulting evidence is conflicting. In a large study from China, the effect of Caesarean section delivery on vertical transmission of HBV was evaluated in 1409 infants born to 1401 HBsAg-positive mothers of whom 61.5% (863 of 1401) had detectable levels of HBV DNA. All the children enrolled completed appropriate immunization against HBV. A lower vertical transmission rate was observed among infants in the group delivered by elective Caesarean section (1.4%) compared with that of those in the vaginal delivery group (3.4%). In the multivariate analysis, elective Caesarean section was beneficial for vertical transmission prevention only in mothers with maternal HBV DNA levels > 200000 IU/mL. In line with this study, two recent systematic reviews with meta-analysis showed that Caesarean section reduced the risk of vertical transmission in infants of HBeAg-positive mothers who did not receive antiviral therapy during pregnancy[10]. Other previous studies had contradictory results regarding the benefit of elective Caesarean section. Overall, there is no robust evidence to support Caesarean section as the mode of choice for the prevention of HBV transmission. The possible beneficial effect of Caesarean section should be weighed against the efficacy of the other well recognised practices for prevention of transmission, (*i.e.* antiviral therapy during pregnancy and passive and active immunoprophylaxis at birth). Thus far, regardless of viraemia, the mode of delivery of mothers with chronic HBV infection should follow the usual obstetric indications and is not influenced by the presence of the infection.

Amniocentesis and other obstetric procedures

Invasive diagnostic procedures during pregnancy, such as amniocentesis, occur before the timing for immunoprophylaxis and may favour the mixing of maternal and foetal blood. Different studies[11-15] conducted before the routine use of HBV viral load testing did not demonstrate an augmented risk for *in utero* infection after amniocentesis in women with chronic infection. In a recent study enrolling 642 consecutive Chinese infants born to HBsAg positive mothers without antiviral exposure and who completed appropriate immunization, 63 infants with amniocentesis were compared with 198 matched infants selected from the remaining 579 infants without amniocentesis. There was a significantly higher vertical transmission rate in infants with amniocentesis than in those without amniocentesis if the maternal HBV DNA levels were ≥ 2×10^6 IU/mL (50% *vs* 4.5%, respectively, $P = 0.006$). On the basis of this result, adequate counselling is advised for HBV-infected women who may necessitate invasive testing (*e.g.*, amniocentesis or chorionic villus sampling) including the possible increased risk for maternal-foetal transmission with HBV viral load ≥ 2×10^6 IU/mL[16].

All the procedures that break the skin and mucosal barrier including foetal scalp electrodes and blood sampling and vigorous suctioning of the newborn's airway at birth should be avoided. The risk of traumatizing the foetal skin is lower with vacuum extraction and forceps, and its use should follow obstetric indications.

Breastfeeding

We identified three major questions concerning breastfeeding and vertical transmission of HBV: (1) Does breastfeeding increase the risk of vertical transmission of HBV? (2) Does breastfeeding interfere with the immune response to vaccine? and (3) Is breastfeeding from HBV-infected mothers on antiviral treatment contraindicated? The role of breastfeeding in the transmission of hepatitis B has been discussed for many years. Examination of relevant studies indicates that there is no evidence that breastfeeding poses any additional risk to infants of HBV carrier mothers[17-19]. The risk of vertical transmission of HBV through breastfeeding is negligible if infants born to HBV-positive mothers who receive the hepatitis B immunoglobulins (HBIG)/hepatitis B vaccine at birth, and the benefits of breastfeeding outweigh any potential risk of infection. HBV infection should not be considered a contraindication to breastfeeding of infants who receive the HBIG and HBV vaccine[20]. Data are insufficient to say whether it is safe or not for the HBV-positive mother to breastfeed if her nipples are cracked and bleeding. Breastfeeding should be temporarily stopped to avoid any

potential exposure to blood, and once nipples are no longer cracked or bleeding, the HBV-positive mother may fully resume breastfeeding.

Wang *et al*[21] have showed that breastfeeding does not interfere with the immune response to the HBV vaccine. A total of 230 babies with HBV immunoprophylaxis at birth were followed up for 1 year in order to measure rates of anti-HBs antibodies at different ages. There were no significant differences in the incidence of immunoprophylaxis failure between breast-fed and formula-fed babies[21]. For mothers who received antivirals during pregnancy, the safety of continuing these drugs after delivery during breastfeeding has been and is a matter of concern and discussion. Although the risk of *in utero* exposure to drugs is likely higher than for infants through breast milk, antivirals are recommended for use during pregnancy but many experts remain concerned about long-term consequences of prolonged antiviral agent exposure in the neonate and of its possible impact on growth and development. However, breastfeeding is advantageous on many issues, especially in low-income countries where formula feeding is not widely available. Furthermore, in human-immunodeficiency setting, antiretroviral treatment could continue during the breast-feeding period in infected women. Only a small quantity of oral nucleoside analogues is secreted in breast milk[22], and the effect on bone growth of exposed children is not significantly different after a follow-up period[23]. In women treated with tenofovir, presence of the drug in breast milk has been reported, but its oral bioavailability is limited, and thus infants are exposed to only small concentrations. Current recommendations by the European Association for the Study of Liver Disease stated that breastfeeding is not contraindicated in HBV-positive mothers on tenofovir-based treatment or prophylaxis.

PREVENTION OF VERTICAL TRANSMISSION OF HBV: MANAGEMENT STRATEGIES DURING PREGNANCY

Maternal screening

The first step to prevent vertical transmission of HBV is to test all pregnant women in the first trimester in order to identify the best management strategy for mothers and the correct immunoprophylaxis schedule for future newborns[24]. In case of positive HBsAg, it is necessary to perform further investigations (hepatitis B core antibody, HBeAg, hepatitis B e antibody, serum aminotransferase levels, quantification of serum HBV DNA, liver imaging) to determine the woman's hepatitis B phase and therefore the possible requirement for treatment during or after pregnancy[25]. In HBsAg negative women with an increased risk of infection (infected partners, infected family members, at risk habits) the evaluation of maternal serological status should also be repeated when entering the hospital at the time of delivery.

In recent years there is a growing interest in new biomarkers of HBV infection, such as covalently-closed circular DNA (cccDNA), hepatitis B core-related antigen, and circulating HBV RNA. cccDNA is a key factor for the persistence of infection and represents a specific marker of replication[26] and was shown to persist in the liver, serum, and peripheral mononuclear cells[27].

Hepatitis B vaccination during pregnancy

Vaccination against HBV during pregnancy is safe and effective[28,29]. There is agreement that pregnant women who are not immune or infected with HBV, whether or not at high risk for HBV infection (as defined by having > one sex partner during the previous 6 mo, a current diagnosis of a sexually transmitted disease, having had an HBsAg-positive sex partner or a recent or current injection drug use), should be vaccinated[16,25]. Following the vaccination, maternal antibodies are passively transferred across the placenta to newborns, although without the active vaccination at birth, its titres rapidly wane over time[28]. Pregnant women can be considered HBV-immune when anti-HBs levels are higher than 10 mIU/mL. Sheffield *et al*[30] have shown that an accelerated vaccination schedule at 0, 1, and 4 mo in high-risk pregnant women is effective and well tolerated.

Hepatitis B immunoglobulin during pregnancy

The rationale behind the possible use of HBIG and/or of antiviral treatment during pregnancy is that up to 10% of infants born to HBV-infected mothers still have HBV infection despite receiving HBIG and HBV vaccine at birth. This suggests that additional interventions during the pre-birth phase could be favourable to decrease

the transmission rate.

HBIG is a purified solution of human immunoglobulin that could be administered to the mother, newborn, or both. When HBIG is administered to pregnant women, the antibodies passively diffuse across the placenta to the foetus. The maternal-foetal diffusion is maximal during the third trimester of pregnancy. Several studies have explored the efficacy of the administration of HBIG to HBV-infected pregnant women [31-34]. Unfortunately, the studies are quite heterogeneous in term of HBIG doses and routes of administration and of definitions of maternal and neonatal infection. A recent Cochrane review found varying effects of maternal antenatal HBIG in preventing vertical transmission of HBV. This review selected 36 trials originated from China including 6044 pregnant women who were HBsAg, HBeAg, or HBV DNA positive. Most of the trials (30/36; 83%) assessed HBIG 200 IU at 28, 32, and 36 wk of pregnancy. Serological signs of hepatitis B infection of the newborns were reported as HBsAg, HBeAg, and HBV DNA positive results at end of follow-up. Although, overall HBIG seemed to impact the HBsAg and HBV DNA status of the newborn, due to low quality evidence found in the review, the authors concluded for the uncertainty of the effect of benefit of antenatal HBIG administration to the HBV-infected mothers on newborn outcomes as compared with no intervention[35].

Antiviral treatment during pregnancy

The use of nucleoside or nucleotide analogues (lamivudine, telbivudine, or tenofovir [36-38]) during the last trimester of pregnancy in highly viraemic, HBeAg positive mothers, in combination with standard infant immune-prophylaxis, has been shown to be effective in further reducing the vertical transmission of HBV[36,37].

Antiviral treatment should be considered based on HBV DNA quantification, and it has been generally suggested in pregnant women with HBV DNA levels of more than 2×10^5 IU/mL. The appropriate time to start and stop antiretroviral drug in pregnant women is still debated. The aim of therapy is to reduce HBV DNA levels below the threshold of transmission or immunoprophylaxis failure at the time of delivery, and for this reason treatment is mainly started around 28 wk to 32 wk of gestation. Earlier may be beneficial and has been suggested for prevention of early placental infection and intrauterine transmission[39]. When the treatment is started only to prevent vertical transmission, it could be discontinued as early as at delivery or, as suggested by the major international societies, prolonged until 12 wk after delivery. While small amounts of drugs are usually present in breast milk, there is a potential risk of maternal hepatitis flare following the end of treatment, most of which are asymptomatic. However, there is no additional benefit in the aspect of hepatitis flare prevention in women who carry on treatment to 4 wk postpartum[40]. Close check of transaminase levels is needed after the end of treatment. Lamivudine[41], telbivudine [42], and tenofovir disoproxil fumarate[43] are the antiretroviral drugs that are considered safe to use during pregnancy. Telbivudine and lamivudine could significantly reduce transmission in infants compared with cases with no treatment, but both drugs have a low genetic barrier to resistance barrier. Therefore, tenofovir disoproxil fumarate is the treatment of choice for HBV-positive mothers because of its potent antiviral activity and high genetic barrier to resistance. Tenofovir alafenamide fumarate is a prodrug of tenofovir that can be administered at a lower dose compared with tenofovir disoproxil fumarate, as its active metabolite could be delivered to the target organs with lower circulating drug levels. The efficacy and safety of tenofovir alafenamide fumarate in HBV-infected pregnant women need to be evaluated before recommending it for use.

Treatment guidelines differ mainly with regard to the type of treatment, the threshold viraemia level, and timing for starting antiviral treatment. Consistency across the different guidelines seems a desirable and achievable target in order to standardise the global approach to mothers with HBV infection and antenatal prevention of vertical transmission.

Indications for treatment including which drug, the threshold of HBV DNA level, when to start, and when to stop treatment, as recommended by the main international scientific societies are summarised in Table 1[44]. Despite the different indications provided by the current guidelines, all societies agree to start antiviral treatment when HBV DNA levels are higher than 2×10^5 IU/mL, regardless of maternal serological status (HBeAg positive or negative).

In 2018, a large, double-blinded randomised placebo-controlled trial of tenofovir disoproxil fumarate given from 28 wk of gestational age to 8 wk postpartum to HBeAg-positive pregnant women with a mean HBV DNA of 10^8 IU/mL in Thailand, plus birth-dose vaccination and HBIG, did not find a significantly lower vertical transmission rate beyond the low rate already achieved in the comparison group that

Table 1 Recommendations for antiviral treatment in pregnant women with chronic hepatitis B virus infection

Societies	Antivirals	HBV-DNA level	When to start treatment	When to stop treatment
American Association for the Study of Liver Diseases[25]	Tenofovir disoproxil fumarate	> 2×10^5 IU/mL	28-32 wk	At birth to 3 mo
European Association for the Study of the Liver[24]	Tenofovir disoproxil fumarate	> 2×10^5 IU/mL	24-28 wk	Up to 12 wk after delivery
Asian Pacific Association for the Study of the Liver[70]	Tenofovir disoproxil fumarate, telbivudine	> 10^{6-7} IU/mL	28-32 wk	At delivery
Chinese Medical Association[71]	Tenofovir disoproxil fumarate, telbivudine, lamivudine	> 2×10^6 IU/mL	24-28 wk	At delivery
National Institute for Health and Care Excellence[72]	Tenofovir disoproxil fumarate	> 10^7 IU/mL	3rd trimester	4-12 wk after birth

HBV: Hepatitis B virus.

was given infant HBIG and HBV vaccination initiated at birth[45]. The study confirmed a significant drop at delivery of HBV DNA for the pregnant women treated with tenofovir. However, all infants received HBV vaccine and immunoglobulin at a mean time of 1.2 and 1.3 h after delivery, and the vertical transmission rate with the administration of HBIG and vaccine in the placebo group was low (2% instead of the expected 12%). Furthermore, mothers with signs of HBV-related liver disease (alanine aminotransferase > 30 IU/L) were excluded and both the tenofovir and the placebo groups consisted of mothers with low viral loads at baseline, possibly impacting the results of the study.

PREVENTION OF VERTICAL TRANSMISSION OF HBV: MANAGEMENT STRATEGIES AT BIRTH

Neonatal immunoprophylaxis: The birth vaccine dose

Post-exposure combined immunoprophylaxis through early administration of the first dose of vaccine and of HBIG is the most effective weapon to prevent vertical transmission of HBV. Without any preventative measures, the risk of vertical transmission for HBeAg-positive and HBeAg negative mothers ranges from 70% to 90% and from 10% to 40%, respectively[46]. The administration of HBV vaccine within 12 h of birth, followed by at least two more doses of vaccine within 6-12 mo[47], is 90%-95% effective in preventing vertical transmission[48,49]. If the administration of HBV vaccine is delayed until 48 h after birth, it would cause significant reduction in neonatal immunoprophylaxis efficacy. The recommendation by the WHO is to provide the first dose of hepatitis B vaccine as soon as possible after birth, preferably within 24 h[50], even in areas where HBV is of low endemicity. The combined approach with hepatitis B vaccine and HBIG at birth is not affordable in most of the endemic low and middle income countries. In these countries, considering the limited resources and the lack of access of HBIG, the WHO identifies HBV vaccination within 24 h of birth as the minimum intervention level and the main strategy to prevent infection[51]. In 2016 the coverage for the three-dose series of hepatitis B vaccine in infancy was estimated to be 84% (compared with 1% in 1990), and birth-dose coverage was estimated to be 39%.

Neonatal immunoprophylaxis: The combined vaccine and hepatitis B immunoglobulin approach

In addition to the HBV vaccination, providing a dose of HBIG at birth to the vaccinated infants can further reduce the risk of transmission, especially in highly viraemic mothers, to less than 5%[52-54]. This was first demonstrated by Wong and collaborators[55] in 1984 in a prospective study enrolling 189 infants who were randomly assigned to receive (1) vaccine at birth and at 1, 2, and 6 mo with seven monthly HBIG injections (100 IU); (2) the same vaccine schedule but only one HBIG injection at birth; (3) only the vaccine, at months 0, 1, 2, and 6; and (4) placebos for both vaccine and HBIG. In all three treatment groups, development of the persistent carrier state was significantly less frequent than in the placebo group (2.9%, 6.8%, 21%, and

73.2%, respectively). Vaccination alone was associated with a remarkable protection toward vertical transmission but was significantly less protective than vaccination plus multiple HBIG injections.

HBIG are obtained from plasma donors with high levels of anti-HBs antibodies. Standard immunoglobulins are not indicated for prevention of vertical transmission of HBV because they contain too low antibody titres against HBV. Timely administration of HBIG and hepatitis B vaccine is critical for interrupting vertical transmission[47]. The Centers for Disease Control and Prevention recommends that the birth dose of HBIG and hepatitis B vaccine be given within 12 h after birth through intra-muscular injection but in an anatomic site different from that of the vaccine[47,56,57]. The earlier the administration of HBIG, the higher is the efficacy of the intervention that is unlikely to exceed the 7th day of birth. After administration of HBV vaccination combined with HBIG, infection can still occur in 2%-10% of HBeAg-positive or highly viraemic mothers[8,45,58]. Failure of the vaccine and immune-prophylaxis regimen or transplacental or intrauterine infection could account for this[8,9,59]. HBeAg-positive mothers and mothers with high circulating levels of HBV DNA (> 10^6 IU/mL) have the highest risk of transmission[8,9]. The dose of HBIG generally used in infants is between 100 and 200 IU, corresponding to 30-40 IU/kg. It is important to note that the availability of HBIG in many countries, especially in those with low and middle income, that also have the higher endemicity is still low. The need for refrigerated storage, short shelf life, and low cost of the product should be addressed in order to make the use of HBIG feasible in all the different settings[60].

Specific indications for immunoprophylaxis according to the HBsAg status of the mother and the weight of the child

According to the Advisory Committee on Immunization Practice of the Center for Disease Control (ACIP-CDC) and the Committee on Infection Diseases of the American Academy of Pediatrics, the choice of the post-exposure immunoprophylaxis schedule is based on the mother's antigenic status (HBsAg) and the birth weight of the child (higher or lower than 2000 g)[47,61,62].

Infants born to HBsAg positive mothers

All newborns born to a mother with HBsAg must receive the birth dose of vaccine and HBIG within 12 h of birth regardless of the birth weight. The completion of HBV vaccine is different according to the birth weight. According to the ACIP-CDC, newborns of mothers with HBsAg test not available during pregnancy but with highly suggestive evidence of HBV infection (presence of HBV DNA, HBeAg-positive, or mother known to be chronically infected with HBV) must be considered as born to HBsAg positive mothers[47].

Infants born to women with unknow HBsAg status

Women with unknown HBsAg status at the time of delivery must be tested as soon as possible. In the meantime, newborns must receive the birth dose of the hepatitis B vaccine within 12 h of birth, regardless of birth weight. If the mother is positive, HBIG should be administered as soon as possible within 7 d of birth. If the mother is negative, the vaccination scheme should be completed as scheduled. In children weighing less than 2000 g, considering the potential reduced immunogenicity of the HBV vaccine in these children, it is recommended to administer HBIG within 12 h of birth even if the maternal status is still unknown. The vaccination schedule should be completed as indicated for HBsAg positive mothers[47].

Infants born to HBsAg negative mothers

The WHO Strategic Advisory Groups of Experts recommends that infants receive the HBV vaccine at birth, preferably within 24 h, but administration up to 7 d after birth followed by two or three additional doses can still be effective[63]. In the case of newborns weighing less than 2000 g, the first dose should be administered after 1 mo of life or at the discharge if this occurs earlier.

Completion of HBV vaccine series after the birth dose

The birth HBV vaccine dose should be followed by completion of a vaccine series. A study from the United States enrolling 17951 mother-infant pairs showed that the number of HBV vaccine doses was associated with risk of infant infection[64]. Overall, vertical HBV infection occurred among 1% of infants who received HBV vaccine and HBIG. Infection was detected in 6.7% (3 of 45 infants) of infants who received < three vaccine doses, compared with 1.1% (97 of 9207 infants) of infants who received ≥ three

doses. The ACIP recommends immunoprophylaxis consisting of hepatitis B vaccine and HBIG within 12 h of birth, followed by completion of an HBV vaccine series.

According to the indications from WHO, if the birth weight is more than 2000 g, the vaccination schedule must be completed with two or three more doses[60], starting within the 2nd month of life and administering the final dose after the 24th week of life (164 d). In case of birth weight less than 2000 g, the birth dose should not be considered as part of the vaccination schedule but three additional doses of vaccine will be required for a total of four, starting when the child has reached 1 mo of age[65, 66]. This recommendation is provided because some studies showed that seroconversion rates may decrease among infants with a birth weight < 2000 g after administration of hepatitis B vaccine at birth. However, within the 1st month of age, all medically stable preterm newborns, regardless of their initial birth weight or gestational age, are as likely to respond to HBV immunization as term and larger infants.

Testing infants for anti-HBs and HBsAg

Newborns to HBsAg positive mother should be tested after 1-2 mo from the final vaccine dose and normally at the age of 9-12 mo, through the evaluation of HBsAg and anti-HBs[67,68]. Test should not be executed before 9 mo of age to avoid detection of passive anti-HBs from HBIG administered at birth and to maximise the probability of detecting late HBV infection. Detection of anti-core antibodies is not recommended in infants born to HBsAg positive mothers because can be passively acquired and detected up to the age of 24 mo[47]. HBsAg negative and vaccinated children with anti-HBs titre greater than or equal to 10 mUI/mL have an adequate protection. If anti-HBs titres < 10 mIU/mL, a fourth additional dose should be administered and the test must be repeated after 1-2 mo. In case of persistence of anti-HBs < 10 mIU/mL after four vaccine doses, two additional doses for a total of six may be administered. The test should be repeated 1-2 mo after the sixth dose. In case of non-response, no further doses are expected[69].

CONCLUSION

Vertical transmission of HBV is the leading mode of acquisition of the infection worldwide. Prevention of vertical transmission is possible in the majority of cases through the correct administration of the birth dose of HBV vaccine and HBIG to the neonate. Strategies are needed to promote implementation of birth-dose vaccination and HBIG in low- and middle-income countries where the prevalence of the infection is at the highest. Breastfeeding should be encouraged as long as the infant receives immunoprophylaxis at birth. Further studies on the use of antivirals (tenofovir alafenamide and tenofovir disoproxil fumarate) during pregnancy are required to increase prevention of HBV infection and their effectiveness in preventing vertical HBV infection when used together with to early active and passive immunoprophylaxis.

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MINIREVIEWS

Endoscopic ultrasound fine needle aspiration *vs* fine needle biopsy for pancreatic masses, subepithelial lesions, and lymph nodes

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Abstract

Endoscopic ultrasound tissue acquisition, in the form of both fine needle aspiration (EUS-FNA) and fine needle biopsy (EUS-FNB), is utilized for pancreatic mass lesions, subepithelial lesions, and lymph node biopsy. Both procedures are safe and yield high diagnostic value. Despite its high diagnostic yield, EUS-FNA has potential limitations associated with cytological aspirations, including inability to determine histologic architecture, and a small quantitative sample for further immunohistochemical staining. EUS-FNB, with its larger core biopsy needle, was designed to overcome these potential limitations. However, it remains unclear which technique should be used and for which lesions. Comparative trials are plagued by heterogeneity at every stage of comparison; including variable needles used, and different definitions of endpoints, which therefore limit generalizability. Thus, we present a review of prospective trials, systematic reviews, and meta-analyses on studies examining EUS-FNA *vs* EUS-FNB. Prospective comparative trials of EUS-FNA *vs* EUS-FNB primarily focus on pancreatic mass lesions, and yield conflicting results in terms of demonstrating the superiority of one method. However, consistent among trials is the potential for diagnosis with fewer passes, and a larger quantity of sample achieved for next generation sequencing. With regard to subepithelial lesions and lymph node biopsy, fewer prospective trials exist, and larger prospective studies are necessary. Based on the available literature, we would recommend EUS-FNB for peri-hepatic lymph nodes.

Key Words: Endoscopic ultrasound fine needle aspiration; Endoscopic ultrasound fine needle biopsy; Pancreatic lesions; Subepithelial lesions; Lymph node biopsy

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Core Tip: Endoscopic ultrasound fine needle aspiration (EUS-FNA) and fine needle biopsy (EUS-FNB) provide two methods for endoscopic ultrasound tissue acquisition for pancreatic mass lesions, subepithelial lesions, and lymph node biopsy. Both methods are safe and provide high diagnostic yield. Prospective comparative trials of EUS-FNA *vs* EUS-FNB primarily focus on pancreatic lesions. EUS-FNB provides diagnostic accuracy with fewer needle passes, and may provide higher diagnostic yield for peri-hepatic lymph nodes.

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INTRODUCTION

As medical and surgical therapeutics continue to evolve, there is a renewed emphasis on timely diagnosis of various illnesses. This mantra certainly holds true in gastrointestinal (GI) diseases, including pancreatic malignancies and GI tumors, where early and specific diagnosis guides management and impacts morbidity and mortality [1]. While cross-sectional imaging can characterize lesions, a tissue diagnosis is often required for a definitive diagnosis prior to therapy[2-5]. Endoscopic ultrasound tissue acquisition (EUS-TA) has improved the ability for tissue diagnosis using a minimally invasive technique. The two modalities for EUS-TA, endoscopic ultrasound fine needle aspiration (EUS-FNA) and endoscopic ultrasound fine needle biopsy (EUS-FNB) vary in technique and utility. Common indications for EUS-TA include the diagnosis and staging of pancreaticobiliary and luminal GI malignancy, and assessing lymphadenopathy associated with luminal GI and lung cancers[6]. Additionally, EUS-TA aids in the evaluation of potentially neoplastic GI subepithelial lesions[6]. Comparative studies on the diagnostic ability of EUS-FNA and EUS-FNB have yielded conflicting results. Here we review prospective comparative data on EUS-FNA *vs* EUS-FNB for pancreatic masses, subepithelial lesions, and lymph node biopsy (Table 1-3).

EUS-FNA

EUS-FNA was first introduced in 1992. It is often combined with rapid onsite evaluation (ROSE) to improve diagnostic ability[7,8]. EUS-FNA is now standard of care for sampling pancreatic solid masses, subepithelial lesions, and lymph nodes, among others. The European Society of Gastroenterology and American Society of Gastroenterology recommend EUS-FNA as first line for diagnosing pancreatic lesions [9-11].

Marked variability exists in EUS-FNA equipment and technique. Several different needle sizes are available including 19 G, 20 G, 22 G, and 25 G. Additionally, variability exists in aspiration technique, including the use of negative pressure suction (used with either a 5 mL or 10 mL syringe) or slow stylet pull. The aspirate from EUS-FNA is often sufficient for cytology and adequate for diagnosis, with diagnostic accuracy ranging from 77% to 95% for pancreatic masses[9,10]. Given its minimally invasive technique and small needle size, EUS-FNA has low rates of morbidity[12].

However, several limitations exist for EUS-FNA which obtains a cytological specimen. EUS-FNA is limited by an inability to obtain histological architecture, and the inability to perform immunohistochemical analysis and molecular profiling. This is of particular importance as certain neoplasms, such as stromal cell tumors and lymphomas, may be difficult to diagnose without histologic samples, as their tissue architecture and morphology are essential for accurate pathologic assessment and histochemical studies[9,13-17]. Furthermore, with the increased attention on personalized or precision medicine in oncology, a sufficient tissue sample to perform next generation sequencing is required. Current National Comprehensive Cancer Network guidelines recommend germline testing for any patient with confirmed pancreatic

Table 1 Prospective comparative trials of endoscopic ultrasound fine needle aspiration *vs* fine needle biopsy for solid pancreatic mass lesions

Ref.	Study design	Number of subjects	Needle size (FNA, FNB)	Diagnostic yield/specimen adequacy (EUS-FNA *vs* EUS-FNB)	Diagnostic accuracy (EUS-FNA *vs* EUS-FNB)	Number of passes needed (EUS-FNA *vs* EUS-FNB)	Comments
Bang *et al*[9], 2012	RCT	56	22 G, 22 G Procore	66.7% *vs* 80% (NS)	N/A	1.61 *vs* 1.28 (NS)	
Aadam *et al*[30], 2015	RCT	73	Variable, variable	78.4% *vs* 91.7% (NS)	67.5% *vs* 83.3% (NS)	N/A	
Tian *et al*[31], 2018	RCT	36	22 G, 22 G ProCore	83.3% *vs* 83.3%	N/A	1.83 *vs* 1.11 (P = 0.049)	
Hedenstrom *et al* [33], 2018	RCT, crossover	68	25G, 22G reverse bevel Wilson Cook	N/A	78% *vs* 69% (NS)	N/A	In a subset of non-pancreatic adenocarcinoma, combined modality (EUS-FNA + FNB) was significantly higher compared to EUS-FNA alone
Oppong *et al*[34], 2020	RCT, crossover	108	Variable, variable Sharkcore	71% *vs* 82% (OR 3.23, sig)	64% *vs* 79% (OR 4.79, sig)	N/A	Shorter sampling time and pathology viewing time with EUS-FNB. Equivalent cost analysis.
Kandel *et al*[35], 2020	RCT, crossover	50	25 G, variable Sharkcore	100% *vs* 86% (NS)	100% *vs* 100%	N/A	Primary outcome of DNA concentration, significantly higher in EUS-FNB than in EUS-FNA
Wang *et al*[26], 2017	Meta-analysis	921	Variable, variable	81.4% *vs* 88.3% (OR 0.57, sig)	84.0% *vs* 87.8% (NS)	Fewer in EUS-FNB	
Li *et al*[27], 2018	Meta-analysis	1382	Variable, variable	82.3% *vs* 89.4% (OR 1.83, sig)	84.3% *vs* 89.6% (OR 1.62, sig)	Fewer in EUS-FNB	

EUS-FNA: Endoscopic ultrasound fine needle aspiration; EUS-FNB: Endoscopic ultrasound fine needle biopsy; RCT: Randomized controlled trial; N/A: Not applicable; NS: Not significant.

cancer using comprehensive gene panels for hereditary cancer syndromes, as well as tumor/somatic gene profiling for patients with locally advanced or metastatic disease to identify mutations that may benefit from anti-cancer therapy. Testing on tumor tissue is preferred; however, cell-free DNA testing can also be considered[18]. There is uncertainty whether EUS-FNA will be able to routinely provide adequate material for these studies[6].

EUS-FNB

In an attempt to overcome the limitations of EUS-FNA, EUS-FNB was first introduced in the early 2000s to obtain tissue specimens as opposed to aspiration-based cytology. With the goal of evaluating tissue core, EUS-FNB provided novel needles for improved diagnostic accuracy.

Table 2 Prospective comparative trials of endoscopic ultrasound fine needle aspiration *vs* fine needle biopsy for subepithelial lesions

Ref.	Study design	Number of subjects	Needle size (FNA, FNB)	Lesions sampled	Diagnostic yield/specimen adequacy (EUS-FNA *vs* EUS-FNB)	Diagnostic accuracy (EUS-FNA *vs* EUS-FNB)	Number of needle passes needed (EUS-FNA *vs* EUS-FNB)	Comments
Kim *et al*[47], 2014	RCT	22	22 G, 22 G Procore	All SELs	20% *vs* 75% (P = 0.01)	N/A	4 *vs* 2 (P = 0.025)	
Iwai *et al*[43], 2017	RCT, crossover	23	Variable, variable Procore	Gastric SELs	73.9% *vs* 91.3% (P = 0.12)	N/A	N/A	Histology positive significantly higher in EUS-FNB for 21 mm-30 mm lesions
Hedenstrom *et al*[48], 2018	RCT, crossover	70	Variable, variable reverse-bevel Wilson-Cook	All SELs	N/A	49% *vs* 83% (P < 0.001)	N/A	Extramural lesions lower sensitivity for EUS-FNA but not EUS-FNB)
Nagula *et al*[49], 2018	RCT	18	Variable, variable Procore	All SELs	83.3% *vs* 75% (NS)	N/A	2 *vs* 2 (NS)	

EUS-FNA: Endoscopic ultrasound fine needle aspiration; EUS-FNB: Endoscopic ultrasound fine needle biopsy; RCT: Randomized controlled trial; N/A: Not applicable; NS: Not significant; SELs: Subepithelial lesions.

Table 3 Prospective comparative trials of endoscopic ultrasound fine needle aspiration *vs* fine needle biopsy for lymph node biopsy

Ref.	Study design	Number of subjects	Needle size (FNA, FNB)	Lymph nodes sampled	Diagnostic yield/specimen adequacy (EUS-FNA *vs* EUS-FNB)	Diagnostic accuracy (EUS-FNA *vs* EUS-FNB)	Number of needle passes needed (EUS-FNA *vs* EUS-FNB)	Comments
Nagula *et al*[49], 2018)	RCT	46	Variable, variable Procore	All lymph nodes	92.9% *vs* 94.4% (NS)	N/A	2 *vs* 2 (NS)	
de Moura *et al*[52], 2020)	Retrospective study of prospectively collected data	209	Variable, variable	All lymph nodes	N/A	78.8% *vs* 83.2% (NS)	N/A	For peri-hepatic lesions, EUS-FNB was significantly more accurate

EUS-FNA: Endoscopic ultrasound fine needle aspiration; EUS-FNB: Endoscopic ultrasound fine needle biopsy; RCT: Randomized controlled trial; N/A: Not applicable; NS: Not significant.

Early models of EUS-FNB utilized Trucut needle biopsy, with a tissue penetrating stylet within an outer cannula. The 19 G Trucut FNB proved more accurate than EUS-FNA for diagnosing lymphomas and stromal tumors, but was limited by mechanical failure when attempting to biopsy pancreatic head masses and duodenal lesions due to the torqued echoendoscope and mechanical friction[16,19]. Newer models, including EchoTip HD ProCore™ (Wilson-Cook Medical Inc., Winston-Salem, NC, United States) is available in 19-25 G, and provides two cutting surfaces, a tip and reverse bevel, to further preserve histological architecture[1]. The reverse or opposing cutting bevel design of the EUS-FNB needle allows for the biopsy of core histopathologic tissue. This

aspect has the potential advantage of improving diagnostic performance, but also allowing a wide range of follow-up testing[20]. Immunohistochemistry, which is required for the diagnosis of autoimmune pancreatitis, lymphoma, and metastasis, can be performed on the tissue core. Furthermore, molecular analysis, which is now standard of care for pancreatic malignancies, can also be performed. Other needles, including SharkCore™ (Medtronic Inc., Sunnyvale, CA, United States) and Acquire™ (Boston Scientific, Malborough, MA, United States), (Figure 1) may achieve even higher diagnostic accuracy[21,22].

Studies have demonstrated high diagnostic yields of core specimens with EUS-FNB with fewer needle passes[23-25]. The potential concern for increased bleeding when using EUS-FNB is offset by the fewer passes required for diagnosis.

EUS-FNA *VS* EUS-FNB: OVERVIEW

Several comparative trials have evaluated EUS-FNA *vs* EUS-FNB. Interpreting the conflicting data is challenging, as trials are plagued by heterogeneity in every stage of comparison. Reported outcomes as well as definition of those outcomes vary between studies. For example, inconsistent use of the term "diagnostic accuracy" and "diagnostic adequacy" creates confusion. Furthermore, heterogeneity exists within equipment use (needle size), and technique (suction *vs* slow pull; specified number of passes). Additionally, designing strong randomized trials is limited by the inability to blind endosonographers, and sometimes cytopathologists, to the type of needle used [26-57].

METHODS

In compiling this review article, we performed a literature search utilizing PUBMED, EMBASE, and Google Scholar for comparative trials of EUS-FNA *vs* EUS-FNB for pancreatic mass lesions, subepithelial lesions, and lymph nodes. A total of 77 articles were identified. Trials were excluded if they were retrospective (n = 26), if they did not directly compare EUS-FNA and EUS-FNB (n = 18), or if they were incomplete manuscripts (n = 6). Any study performed on a variety of mass lesions without subcategories for the aforementioned groups was also excluded (n = 4).

PANCREATIC LESIONS

Pancreatic adenocarcinoma is characterized by a poor prognosis, with a 5-year survival rate of 5%-6%[27]. Pancreatic adenocarcinoma may be difficult to differentiate from other pancreatic mass lesions based on cross-sectional imaging and abdominal ultrasound[2-5]. The reported sensitivity of EUS in the detection of pancreatic cancer is between 94% and 100%[28]. Compared to computed tomography (CT), EUS can detect up to 14% of pancreatic tumors that were not visualized on CT, especially tumors smaller than 20 mm[11]. As such, EUS is currently the standard method for tissue diagnosis of pancreatic masses[11].

Tissue sampling of pancreatic mass lesions by EUS is vital in diagnosis. Several sampling approaches are possible depending on the location of the pancreatic mass lesion. A trans-duodenal approach may be optimal for lesions in the pancreatic head, while the transgastric approach is more appropriate for lesions in the pancreatic body and tail. Bang *et al*[29] proposed an algorithm for needle selection based on anatomical site; a 25 G needle for the trans-duodenal approach and a 22 G or 25 G for all other punctures.

Comparative trials of EUS-FNA *vs* EUS-FNB for pancreatic mass lesions focus mostly on safety, diagnostic accuracy, sample adequacy for diagnosis and further testing (Table 1).

Randomized controlled trials of pancreatic masses with EUS-FNA or EUS-FNB

Bang *et al*[9] performed the earliest randomized controlled trial (RCT) comparing EUS-FNA and EUS-FNB. The study randomized 56 patients to receive either EUS-FNA 22 G or EUS-FNB 22 G ProCore for pancreatic mass lesions, with the primary outcome being the number of passes required to establish a diagnosis with ROSE. They found no significant difference in the median number of passes required to establish on-site

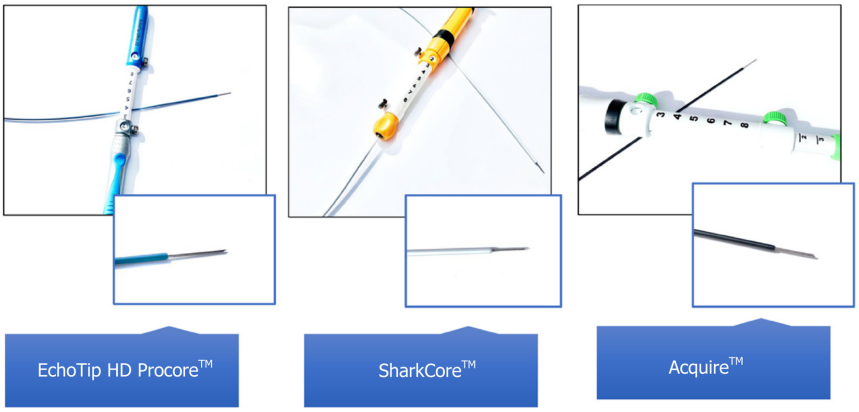


Figure 1 Fine needle biopsy needles.

diagnosis, and overall similar rates of diagnosis were achieved within 3 passes (100% EUS-FNA, 89% EUS-FNB). Incomplete diagnosis by EUS-FNB was due to diagnostic failure in two patients, and technical failure in 1 patient. Procedural complications among the two techniques were similar (one patient with post-procedural abdominal pain in the EUS-FNA cohort, and one patient with pancreatitis in the EUS-FNB cohort). With regard to secondary outcomes, EUS-FNA had a higher proportion of samples with histologic core tissue present (100% *vs* 88.3%, not-significant) but EUS-FNB had a higher percentage of histologic core tissue optimal for histochemical testing.

It is noteworthy that their technique varied from subsequent trials in several respects, and perhaps limited the study's generalizability. First, ROSE was carried out for all specimens, thereby possibly preferentially inflating the diagnostic ability of EUS-FNA. Additionally, they utilized an earlier model of FNB, the 22 G Echotip ProCore™ devise. Lastly, they utilized fewer needle movements for the EUS-FNB cohort (only 4 movements to and fro).

A subsequent larger RCT by Aadam *et al*[30] similarly showed no difference in diagnostic yield or specimen adequacy between EUS-FNA and EUS-FNB in patients with pancreatic lesions.

A 2018 RCT by Tian *et al*[31] of 36 patients similarly showed no superiority in diagnostic accuracy between EUS-FNA and EUS-FNB; although they did find a difference in the number of passes needed to make a diagnosis. Similar to Bang *et al*[9], patients were randomized to either EUS-FNA or EUS-FNB ProCore for solid pancreatic masses, although ROSE was not performed on any of the specimens. For the primary outcome of diagnostic yield, the authors found identical results (83%). However, among their secondary outcomes, EUS-FNB required fewer passes to make a diagnosis (1.11 *vs* 1.83, $P < 0.05$). It is noteworthy that a smaller percentage of their cohort were diagnosed with pancreatic adenocarcinoma (66.7%) compared to other trials. There were no complications in either cohort in their study.

Similar findings were also demonstrated in a larger, more recent RCT performed by Chen *et al*[32]. The authors randomized 235 patients with pancreatic mass lesions to EUS-FNA + ROSE (n = 120) *vs* EUS-FNB (22 G or 25 G Fork-tip needle, n = 115). For the primary outcome of diagnostic accuracy, the authors found no difference (92.2% *vs* 93.3%, respectively). However, among the secondary outcomes, EUS-FNB was associated with fewer needle passes to make a diagnosis compared to EUS-FNA + ROSE (2.3 *vs* 3.0) and decreased procedure time (19.3 min *vs* 22.7 min). There were no adverse events in the EUS-FNB cohort, and three adverse events in the EUS-FNA cohort (2 pancreatitis, 1 bleeding).

Crossover trials

In contradistinction to the aforementioned articles, in several trials patients underwent both EUS-FNA and EUS-FNB in a crossover study design, thereby allowing direct comparison between specimen procurement in the same patients and providing an internal control. Hedenstrom *et al*[33] randomized 68 patients with a pancreatic mass to receive either EUS-FNA (25 G) followed by EUS-FNB (22 G), 1 pass each, or vice versa. A reverse bevel EUS-FNB 22 G needle was used (Wilson-Cook Medical) and further passes were performed by alternating the two needles. They utilized similar

suction (10 cc) and fanning techniques for both EUS-FNA and EUS-FNB. ROSE was carried out for the majority of both EUS-FNA and EUS-FNB samples. The primary outcome of diagnostic accuracy was not significantly different between the two methods of tissue acquisition. No adverse events were recorded.

Utilizing a newer model of EUS-FNB Fork tip (SharkCore™ FNB Needle), Oppong *et al*[34] randomized 108 patients with pancreatic mass lesions to EUS-FNA and then EUS-FNB, 3 passes each, or vice versa. The primary endpoint was diagnostic performance for malignancy (malignant yes/no), compared to a gold standard of unequivocal malignant pathology obtained by EUS sampling, surgical resection, or alternative biopsy. For non-operated patients, clinical and radiological disease progression consistent with malignancy at 6-mo follow-up was required. The authors found increased sensitivity for the diagnosis of malignancy with EUS-FNB compared to EUS-FNA (82% *vs* 71%). The study was unique in that it also assessed procedural time and pathology viewing time, both of which were significantly shorter for EUS-FNB (710 s *vs* 759 s, $P = 0.001$; 188 s *vs* 332 s, $P < 0.001$, respectively). The authors performed a cost-analysis and found no significant difference; however, they analyzed only materials used and did not factor in operational/labor time. The authors reported four serious adverse events (2 cholangitis, 1 pancreatitis, 1 abdominal pain), but did not specify which cohorts the patients belonged to.

Other studies have utilized alternative endpoints to diagnostic accuracy or adequacy. As discussed previously, obtaining a diagnosis for pancreatic adenocarcinoma may still require further testing for personalized medicine, and therefore additional tissue may be required. Kandel *et al*[35] performed a RCT of 50 consecutive patients to assess adequacy for genomic profiling. In their study, they randomized patients to EUS-FNA followed by EUS-FNB (or vice versa) in a randomized order. They also utilized the SharkCore™ FNB needle. The first pass with each needle was used for histology, and subsequent passes were used to collect DNA. They found that EUS-FNB yielded significantly higher mean DNA concentrations compared to EUS-FNA (5.930 μg/mL *vs* 3.365 μg/mL, $P = 0.01$).

These findings have unclear clinical significance, since despite the quantitative difference in DNA acquired, both acquisition techniques yielded sufficient DNA for next generation sequencing (approximately 10 ng/μL). Furthermore, it is noteworthy that the EUS-FNA utilized a smaller needle (25 G) compared to both EUS-FNB needles (19 G or 22 G). This was likely done to maximize diagnostic accuracy, which was similar in both cohorts (100% final diagnosis in both), but may come at the expense of the DNA quantity acquired.

Systematic reviews and meta-analyses

Several systematic reviews and meta-analyses have attempted to summarize the conflicting data on pancreatic lesions. However, heterogeneity in the studies included and outcomes measured further perpetuate the confusion.

In 2017, Wang *et al*[26] performed a meta-analysis on 8 RCTs to determine diagnostic accuracy. Significant variability existed within needle size and suction technique between the trials. For diagnostic accuracy, they found no significant difference between EUS-FNA (84%) and EUS-FNB (88%, OR 0.72; 95%CI: 0.49-1.07). Among the 5 trials that reported specimen adequacy, and the four trials reporting the number of needle passes required, EUS-FNB demonstrated superiority (OR 0.57, 95%CI: 0.37-0.89; and OR 0.86, 95%CI: 0.45-1.26, respectively). Among the five studies that reported adverse events, the rates were low and not significantly different between the two groups (2/313 in the EUS-FNA group, and 4/311 in the EUS-FNB group), and specific complications were not mentioned.

One year later, in 2018, Li *et al*[27] performed a meta-analysis with the same 8 RCTs, and included an additional 3 RCTs, and yielded different results. They found that EUS-FNB had significantly better specimen adequacy (OR 1.83, 95%CI: 1.27-2.64), and higher diagnostic accuracy (OR 1.62, 95%CI: 1.17-2.26) than EUS-FNA, again with fewer needle passes (MD -0.69, 95%CI: -1.18 to -0.2). There was no difference in complications or technical success.

However, a larger 2019 meta-analysis by Facciorusso *et al*[20] of 27 RCTs found different results. They evaluated diagnostic accuracy, and found no significance difference between needle type (EUS-FNA or EUS-FNB) or needle size. The authors summarized the adverse events as rare among their studies; however, most studies did not itemize the etiology of the adverse events. The only studies that specifically reported bleeding episodes, all reported bleeding in the EUS-FNA cohort. Of note, the authors performed a network meta-analysis technique, thereby utilizing both direct RCT (EUS-FNA *vs* EUS-FNB) as well as indirect evidence (RCT of EUS-FNA *vs* EUS-FNA, or EUS-FNB *vs* EUS-FNB) and then extrapolated the data. Only 14 of the 27 trials

included were actually EUS-FNA *vs* EUS-FNB. As such, their results should be interpreted with caution.

Summary of pancreatic mass studies

Conflicting data exist among prospective studies evaluating the superiority of different EUS-TA techniques. Taken together, both methods provide overall high, and comparable, diagnostic accuracy and specimen adequacy for diagnosis. Adverse events, including bleeding, are rare in both techniques, with pancreatitis being the most common adverse event. Multiple trials have demonstrated that fewer passes are required for EUS-FNB compared to EUS-FNA. The ramifications of this, with the resulting decreased procedural time and likely fewer adverse events, may prove beneficial when applied broadly, but larger trials are required for further elucidation. Additionally, clinical benefit from the increased quantity of tissue obtained remains unclear, if standardized testing and next generation sequencing can be performed on all samples.

SUBEPITHELIAL LESIONS

Subepithelial lesions (SELs) of the GI tract are tumors that originate from the muscularis mucosa, submucosa, or muscularis propria[36]. Initial management of SELs focuses on proper diagnosis and determination of malignant potential, to guide further resection recommendations. EUS is the most accurate imaging method for evaluating SELs of the GI tract[37-39], because it can delineate the individual histologic layers and likely site of tumor origin. Certain SELs have a distinct endoscopic appearance, such as lipomas, duplication cysts, and ectopic pancreas, and endoscopic appearance may be considered diagnostic[36]. However, endoscopic appearance alone is not sufficient for diagnosis in many cases, such as hypoechoic and heterogeneous lesions from the submucosal and muscularis propria, and tissue acquisition is often required. Standard biopsy forceps and jumbo biopsy forceps (bite on bite technique) have low diagnostic yield[40,41].

EUS-FNA is the most widely used method for obtaining SEL tissue arising from the submucosal and muscularis propria layer[36]. However, the diagnostic accuracy of EUS-FNA is variable, ranging from 34% to 93%[39,42]. Additionally, the amount of cytological material obtained by EUS-FNA is often insufficient for the immunohistochemical staining required to differentiate different SELs[43].

Comparative trials

There are few prospective comparison trials of EUS-FNA and EUS-FNB focused solely on SELs, although several larger prospective trials contained cohorts of SELs (Table 2). We excluded trials that did not perform subgroup analysis on this SEL subgroup in isolation[30,44-46].

The first RCT focused solely on SELs was performed by Kim *et al*[47] in 2014. The authors randomized 22 patients with GI SELs of all types to either EUS-FNA (n = 10) or EUS-FNB (n = 12, ProCore). The patients did not receive both methods of tissue acquisition. The cohort was comprised of mostly gastric SELs (17/22), and mainly arising from the muscularis propria (20/22). The needle size was dependent on tumor diameter at the time of EUS, with a 22 G needle used if the tumor was estimated to be < 30 mm, and 19 G used if the tumor was > 30 mm. The authors utilized the unique endpoint of the number of passes required to obtain macroscopically optimal core samples. Since ROSE was not carried out at all sites, the endoscopist immediately inspected the material for the presence of tissue core, defined as whitish pieces of tissue with apparent bulk. If present, no further passes were obtained. However, if absent, the endoscopist proceeded with an additional pass with a maximum of 3 passes. If the sample still did not contain macroscopic tissue core, the number of passes was recorded at 4, and the patient crossed over to the other cohort. The authors found that the median number of needle passes required to obtain macroscopically optimal core sampled by EUS-FNB was significantly lower than that by EUS-FNA (2 *vs* 4, P = 0.025). Despite being macroscopically defined as optimal core samples, the core samples were suboptimal for microscopic analysis in three cases. Overall, the rates of obtaining macroscopically and histologically optimal core samples with EUS-FNB (92% and 75%, respectively) were superior to EUS-FNA (30% and 20%, respectively). No technical difficulties were encountered, and one patient in the entire cohort developed post-procedural bleeding which was managed conservatively. A limitation of the study design was lack of blinding of the endoscopist who assessed the primary

endpoint.

A follow-up study by Iwai *et al*[43] in 2017, focused solely on gastric SELs arising from the muscularis propria and randomized 24 patients to receive either EUS-FNA followed by EUS-FNB or vice versa. The two needles were used alternatively to puncture the same lesion with a total of four punctures per session. Similar to Kim *et al*[47], needle size was dependent on tumor size on EUS, and the ProCore needle was used for all EUS-FNB. The primary outcome was diagnostic yield. The authors found that the rate of correct diagnosis on immunohistochemical staining tended to be higher for EUS-FNB (91.3%) than for EUS-FNA (73.9%, P = 0.120), although this failed to reach statistical significance. When sub-characterized by tumor size, they found that EUS-FNB had significantly higher rates of positive histology among tumors 21-30 mm. The study was limited by sample size and was underpowered, as several of their findings trended towards significance.

A larger 2018 RCT performed by Hedenstrom *et al*[48] similarly found superiority of EUS-FNB to EUS-FNA for SELs, utilizing the reverse bevel ProCore EUS-FNB needle. The study randomized 70 patients with GI SELs to dual sampling with EUS-FNA and EUS-FNB in an alternating fashion until the yield was regarded as satisfactory by the cytotechnician, with a maximum of six passes. Similar to Iwai *et al*[43], in the absence of ROSE, gross examination was performed by the endoscopist. The cohort consisted of mostly gastric SELs (66/70). The study found significantly higher overall diagnostic accuracy for EUS-FNB than EUS-FNA (83% *vs* 49%, P < 0.001). A trend of lower sensitivity of EUS-FNA for extramural lesions compared to intramural lesions was also observed, a trend that did not exist for EUS-FNB. The authors hypothesized that this may be related to increased mobility of extramural lesions, preferentially affecting EUS-FNA diagnostic accuracy. The characterization of intramural and extramural was based on appearance at EUS. The authors reported few adverse events.

These findings are in contrast to Nagula *et al*[49] who found in the SELs cohort (n = 18) that there was no significant difference in diagnostic yield between EUS-FNB ProCore and EUS-FNB (EUS-FNB 75% *vs* EUS-FNA 83.3%, P = 0.754).

LYMPH NODES

Lymphadenopathy may arise from many different etiologies, ranging from benign inflammatory or infectious, to malignant etiologies. Evaluation of lymphadenopathy must include tissue sampling, as lymph node size has demonstrated poor specificity for differentiating malignant from benign lymphadenopathy[50,51]. Clarifying the malignant potential of lymphadenopathy is essential for clinical management[52].

The modality for sampling lymph nodes depends on anatomic location. For mediastinal lymph node sampling, EUS-TA is safer and less invasive compared to alternative techniques[10]. Additionally, for abdominal lymph nodes, EUS-sampling is successful in 92% of patients[53].

EUS-TA for lymph nodes is typically performed with EUS-FNA. However, the sensitivity of EUS-FNA for providing material for cytological evaluation is suboptimal, with reported rates of 88%-96%[54]. The suboptimal results are often attributed to damaged lymph node architecture[51,54]. This limitation of EUS-FNA is important in the evaluation of lymphadenopathy of unknown etiology, where the differential diagnosis includes lymphoma, metastasis, mycobacterial infection, and sarcoidosis, and core biopsy with preservation of lymph node architecture is particularly important for diagnostic purposes[53,55,56].

Comparative trials

We found no published prospective RCTs of EUS-FNA *vs* EUS-FNB for only lymph node biopsy. In the large RCT by Nagula *et al*[49] mentioned above, the subgroup of lymph node biopsies (n = 46) found no difference between EUS-FNA and EUS-FNB Procore in diagnostic yield (92.9% *vs* 94.4%) or number of passes needed to make a diagnosis (median 2, P = 0.43) (Table 3).

De Moura *et al*[52] performed a prospective study comparing EUS-FNA *vs* EUS-FNB exclusively for lymph node diagnosis. The authors performed an analysis on a prospectively collected database of 209 patients undergoing either EUS-FNA (n = 108) or EUS-FNB (n = 101) to evaluate lymph nodes. No predefined protocol was used in the study, and as such several different EUS-FNB needles were used including Acquire, SharkCore, and ProCore. The cohort consisted mostly of peri-hepatic lymph nodes (60%) followed by peri-pancreatic (10.4%) and mediastinal (10.4%), and were mostly accessed *via* a transgastric approach (45%). The pathology of most specimens

was benign (61%). Their primary outcome was diagnostic yield from cytological and histological analysis with and without immunohistochemical staining.

Overall, the authors found similar diagnostic accuracy between EUS-FNA and EUS-FNB (78.8% *vs* 83.2%, $P = 0.423$). However, the specificity for EUS-FNB demonstrated significant superiority (100% *vs* 93.62%, $P = 0.01$). In the subgroup analysis, EUS-FNB showed significantly higher sensitivity and specificity for abdominal lymph nodes. The diagnostic accuracy tended to be greater in the EUS-FNB cohort, but this failed to reach statistical significance. Following further analysis of lymph node location, EUS-FNB was associated with significantly higher sensitivity, specificity, and overall diagnostic accuracy for peri-hepatic lesions (88.9% *vs* 70.5%, $P = 0.038$).

Taken together, the study forms an important backdrop for further research, and argues for consideration of EUS-FNB over EUS-FNA for lymph node biopsy, specifically for peri-hepatic lesions.

UTILIZING BOTH TECHNIQUES

Additional studies have assessed the additive benefit of sampling lesions with both EUS-FNA and EUS-FNB. One such study by Hedenstrom *et al*[33] found that EUS-FNA/FNB compared to EUS-FNA alone had a higher diagnostic sensitivity for pancreatic tumors (89% *vs* 69%, $P = 0.02$), but not for pancreatic adenocarcinoma. However, compared to the diagnostic accuracy of EUS-FNB in isolation, Keswani *et al*[57] found no additional diagnostic accuracy by including EUS-FNA for pancreatic adenocarcinoma.

CONCLUSION

Endoscopic ultrasound tissue acquisition is routinely utilized in the evaluation of pancreatic mass lesions, subepithelial lesions, and lymph node biopsies. Ongoing confusion surrounds the ideal modality for EUS-TA, whether by EUS-FNA or EUS-FNB. While more robust comparative clinical trials exist for pancreatic lesions compared to subepithelial lesions and lymph nodes, the data continue to be mixed. Randomized controlled trials with homogenous populations and homogenous sampling protocols are needed in order to truly understand which needle is superior.

Based on the literature reviewed in this article, the authors conclude the following: EUS-FNA and EUS-FNB both provide high diagnostic accuracy, with low technical failure and adverse events, and thus either needle can be utilized for EUS-TA of pancreatic lesions, subepithelial lesions, and lymph nodes. In our experience we prefer FNB with a new generation needle as it allows us fewer passes of the needle, allows us to forgo ROSE which adds significant time and resources to a procedure, and gives a sample suitable for molecular testing. When increased quantity of DNA is desired for next generation sequencing, the utilization of EUS-FNB should be considered. For extramural subepithelial lesions, the utilization of EUS-FNB should be considered. Despite the dearth of prospective literature, we would recommend EUS-FNB for lymph node biopsy, specifically for peri-hepatic nodes.

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ORIGINAL ARTICLE

Basic Study

Metal-organic framework IRMOFs coated with a temperature-sensitive gel delivering norcantharidin to treat liver cancer

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Abstract

BACKGROUND

Norcantharidin (NCTD) is suitable for the treatment of primary liver cancer, especially early and middle primary liver cancer. This compound can reduce tumors and improve immune function. However, the side effects of NCTD have limited its application. There is a marked need to reduce the side effects and increase the efficacy of NCTD.

AIM

To develop a nanomaterial carrier, NCTD-loaded metal-organic framework IRMOF-3 coated with a temperature-sensitive gel (NCTD-IRMOF-3-Gel), aiming to improve the anticancer activity of NCTD and reduce the drug dose.

METHODS

NCTD-IRMOF-3-Gel was obtained by a coordination reaction. The apparent characteristics and *in vitro* release of NCTD-IRMOF-3-Gel were investigated. Cell cytotoxicity assays, flow cytometry, and apoptosis experiments in mouse hepatoma (Hepa1-6) cells were used to determine the anti-liver cancer activity of NCTD-IRMOF-3-Gel in *in vitro* models.

RESULTS

The particle size of NCTD-IRMOF-3-Gel was 50-100 nm, and the particle size distribution was uniform. The release curve showed that NCTD-IRMOF-3-Gel had an obvious sustained-release effect. The cytotoxicity assays showed that the free drug NCTD and NCTD-IRMOF-3-Gel treatments markedly inhibited Hepa1-6 cell proliferation, and the inhibition rate increased with increasing drug concentration. By flow cytometry, NCTD-IRMOF-3-Gel was observed to block the Hepa1-6 cell cycle in the S and G2/M phases, and the thermosensitive gel

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nanoparticles may inhibit cell proliferation by inducing cell cycle arrest. Apoptosis experiments showed that NCTD-IRMOF-3-Gel induced the apoptosis of Hepa1-6 cells.

CONCLUSION

Our results indicated that the NCTD-IRMOF-3-Gel may be beneficial for liver cancer disease treatment.

Key Words: Norcantharidin; Metal-organic frameworks; IRMOF-3; Temperature-sensitive gel; Drug delivery; Liver cancer

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Core Tip: Norcantharidin (NCTD) is suitable for the treatment of primary liver cancer, especially early and middle primary liver cancer. However, the side effects of NCTD have limited its application. Therefore, we established a liver-targeting therapy in which NCTD is loaded into IRMOF-3 coated with a thermosensitive gel, which can be efficiently delivered to liver cancer cells and slowly released. The results demonstrate that this thermosensitive gel-encapsulated IRMOF-3 has great advantages as an antitumor drug carrier and provides some ideas for passive targeting therapy of tumors.

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INTRODUCTION

Liver cancer has the characteristics of a high incidence, poor prognosis, and high mortality. The latest World Health Organization data show that the global incidence rate of liver cancer is ranked fifth among malignant tumors, and the incidence rate is ranked third. China is a country with a high incidence of liver cancer and hepatitis B[1,2]. At present, surgical resection[3,4], drug chemotherapy[5,6], nanotechnology[7], and interventional therapy[8] are the main treatment methods for liver or other cancers. Among many chemotherapeutic drugs, norcantharidin (NCTD) has strong antitumor activity and can inhibit a variety of tumors including gastrointestinal cancer[9], malignant lymphoma[10], lung cancer[11], and liver cancer[12].

NCTD was synthesized by removing the 1,2-methyl group from cantharidin, which was extracted from the cantharides of Coleoptera. Compared with cantharidin, NCTD exhibits not only significantly improved anticancer effect but also a great reduction in renal toxicity and strong irritation to the urinary system[13]. The clinical use of NCTD is mainly based on tablets and injections, and this drug has unique advantages in the treatment of cancer. However, the side effects of NCTD have limited its application [14]. First, compared to cantharidin, the toxicity of NCTD is reduced to a large extent but still has a certain degree of urinary system toxicity, and organ toxicity occurs with large doses or long-term use, so there is a strict limit on the maximum dosage of NCTD in the clinic[15]. Second, NCTD is rapidly distributed in various tissues after absorption when administered to mice by gavage. The concentration of NCTD peaks in liver and cancer tissues 15 min after administration. However, this concentration significantly decreases 6 h after administration. Most NCTD is excreted through the kidney within 24 h, with little accumulation in the body. The elimination speed of NCTD from the body is fast, which reduces the compliance of patients with medication[16]. In addition, NCTD is widely distributed in the body after oral administration, and is less distributed in the liver tissue due to its fast elimination speed, which not only reduces its efficacy but also increases the toxicity to other organs[17]. Third, most NCTD injections used in the clinic are sodium salt, with a pH value of approximately 9.0, which makes it highly irritating[18].

In recent years, a large number of studies have been carried out to reduce the side effects and increase the efficacy of NCTD[19-22]. This project aimed to develop a multifunctional metal-organic framework (IRMOF-3) that can play an important role in drug carrying and delivery. Because of the special topological structure of IRMOF-3, drugs can be loaded into the spatial structure to the maximum extent, and it plays great role in controlled release[23-26]. However, when NCTD-IRMOF-3 enters the body, burst release is caused due to endocytosis or gastrointestinal absorption. Therefore, we established a liver-targeting therapy in which NCTD is loaded into IRMOF-3 coated with a thermosensitive gel (NCTD-IRMOF-3-Gel), which can be efficiently delivered to liver cancer cells and slowly released. In this study, NCTD-IRMOF-3-Gel was prepared, and the *in vitro* targeting behavior was explored. It was shown that the combination of IRMOF-3 and the thermosensitive gel could decrease the toxicity and increase the bioavailability of NCTD, representing an effective method for the chemotherapy of liver cancer. This study lays a foundation for the liver-targeting ability of NCTD-IRMOF-3-Gel. The results demonstrate that this thermosensitive gel-encapsulated IRMOF-3 has great advantages as an antitumor drug carrier and provides some ideas for passive targeting therapy of tumors.

MATERIALS AND METHODS

Materials

All of the chemicals used were of analytical grade. N,N-Dimethylformamide (DMF), dichloromethane (CH_2Cl_2) (both Tianjin Fuyu Fine Chemical Co., Ltd., Tianjin, China), zinc acetate dihydrate ($Zn(OAc)_2.2H_2O$) (Kaitong Chemical Industry Co. Ltd., Tianjin, China), and 2-amino-terephthalic acid (NH_2-BDC, $C_8H_7NO_4$) (Henghua Technology Co., Ltd., Jinan, China) were used to prepare nanosized IRMOF-3.

Preparation of NCTD-IRMOF-3-Gel

First, $Zn(OAc)_2.2H_2O$ (4 mmol) and NH_2-BDC (1 mmol) were completely dissolved in 10 mL and 15 mL DMF, respectively. Then the zinc salt solution was quickly poured into the ligand solution at room temperature (25 °C) to form a milky white precipitate. After magnetic stirring for 1 min and centrifugation for 5 min (12000 r/min), the supernatant was removed and the precipitate was obtained. The deposit was washed three times with DMF (removing unreacted raw materials) and soaked for 3 d with CH_2Cl_2 (removing DMF), with the solvent replaced once per day. Next, the deposit was centrifugally filtered and dried under natural conditions. Then, the samples were activated under vacuum for 12 h at 100 °C, and IRMOF-3 was obtained. For the encapsulation studies, 30 mg NCTD and 10 mg nanoIRMOF-3 were accurately weighed in a 5 mL volumetric flask, and 80% alcohol solution was added. The suspension was stirred for 72 h at room temperature. NCTD-loaded nanoIRMOF-3 (NCTD-IRMOF-3) was then collected by centrifugation and vacuum-dried at room temperature. NCTD-IRMOF-3 (15 mg) was accurately weighed, 3 mL freeze-dried protective agent (4% mannitol and 2% poloxamer) was added, and the mixture was fully dissolved. A small amount of supernatant was collected, frozen in a vial at -40 °C for 24 h, and then frozen in a vacuum freeze dryer for 30 h to obtain the freeze-dried product. NCTD-IRMOF-3 was then dispersed into a thermosensitive gel solution at room temperature to form a dispersion of nanoparticles. When the NCTD-IRMOF-3 nanoparticle dispersion was injected into the body or heated to 37 °C, the NCTD-IRMOF-3-Gel gel could be formed.

Characterization

Physical characterization was performed by powder X-ray diffraction (PXRD) analysis (Phillips Xpert Pro MPD diffractometer with Cu Kα radiation λ = 1.5418 nm at 40 kV and 50 mA). Scanning electron microscopy (SEM) images were obtained using a Quanta 200F (FEI Sirion SEM), confirming the regular shape and nanosize of the particles. Nitrogen adsorption/desorption isotherms and pore size distributions were measured using an MFA-140 system (Beijing Builder Electronic Technology Co., Ltd., Beijing, China). The particle size distribution was measured by a Zetasizer Nano-ZS90 Laser particle size analyzer (Malvern Instruments Co., Ltd., Malvern, United Kingdom).

NCTD release assay

In vitro studies of the release of NCTD from NCTD, NCTD-IRMOF-3, and NCTD-

IRMOF-3-Gel were carried out using dialysis bags (Sigma, St. Louis, MO, United States) soaked in double-distilled water for 12 h. Freeze-dried NCTD, NCTD-IRMOF-3, and NCTD-IRMOF-3-Gel suspensions were added into a dialysis bag, which then was placed in 50 mL phosphate-buffered saline (pH 5.0) to maintain sink conditions and shaken at 100 rpm in a constant-temperature shaker (SHAB; Donglian Electric Technique Co. Ltd., Harbin, China) at 37 °C. Subsequently, 2 mL release medium was withdrawn at regular intervals, and fresh release medium was added to maintain a constant volume. Every trial was repeated three times. The samples were analyzed using high-performance liquid chromatography, and the control experiments were similarly performed using the same proportions to investigate drug release.

In vitro examination

MTT assay for cytotoxicity detection: The mouse hepatoma (Hepa1-6) cell line (Beijing Boyu Kangtai International Biological Technology Co., Ltd., Beijing, China) used in this experiment was maintained in Roswell Park Memorial Institute-1640 medium (HyClone; Thermo Fisher Scientific, Inc., Waltham, MA, United States) supplemented with 10% heat-inactivated fetal calf serum (Sijiqing Tianhang Biological Science and Technology Co., Ltd., Hangzhou, China), 105 U/L penicillin G, and 100 mg/L streptomycin in a CO_2 incubator at 5% CO_2 and 37 °C.

The cells were plated in 96-well cell culture plates, and different concentrations of NCTD (0-80 μg/mL) were added to the complete cell culture medium. Then, 10 concentrations of NCTD-IRMOF-3 and NCTD-IRMOF-3-Gel were prepared (0-80 μg/mL). After 24, 48, 72, and 96 h of incubation, chemosensitivity was evaluated using thiazolyl blue tetrazolium bromide (MTT reagent, 98%; Wuhan Baodu DE Co., Ltd., Wuhan, China) in complete cell culture medium (5 μg/mL). Then, 20 μL MTT reagent was added to each well and incubated for 4 h, and the mitochondrial aldehyde dehydrogenase from the viable cells subsequently reduced the yellow, water-soluble MTT reagent to water-insoluble blue formazan crystals, which were dissolved by adding 150 μL dimethyl sulfoxide to each well. The absorbance of the dissolved formazan blue dye was measured at 490 nm using a BioTex microplate reader (American Power Instruments Co., Ltd., Wilmington, MA, United States), and the cell viability calculations were performed.

Flow cytometry assay of the effects on the Hepa1-6 cell cycle: The cells were placed in 6-well cell plates and cultured for 24 h in a CO_2 incubator at 5% CO_2 and 37 °C. Then, NCTD-IRMOF-3-Gel at different concentrations (10-40 μg/mL) was added to each well and incubated in an incubator for 24 h. The cells were digested with trypsin (Shanghai Beyotime Biotechnology Co., Ltd., Shanghai, China) and centrifuged at 1500 rpm for 5 min. The supernatant was removed from the solution and washed twice with phosphate-buffered saline (PBS). Then the precooled 75% ethanol solution was added to each well and fixed at 4 °C for 12 h. The solution was centrifuged at 1500 rpm for 5 min to remove the supernatant and resuspended in PBS. Finally, the prepared solution (including 0.5 mL of PBS, 25 μL propidium iodide staining solution and 10 μL ribozyme A) was added to each well and incubated at 37 °C for 30 min. The cells were filtered using a 35 μm cell filter and detected by flow cytometry (Becton, Dickinson and Company, Franklin Lakes, NJ, United States).

Apoptosis experiment: Apoptosis was detected by Annexin V-FITC/PI double staining. K562 cells in the logarithmic growth phase were inoculated into 24-well culture plates at 1×10^5/well. NCTD-IRMOF-3-Gel, NCTD-IRMOF-3 and NCTD were added at two different concentrations (25 μg/mL and 50 μg/mL). After 48 h, the cells were washed with PBS three times, and 1 μL propidium iodide and 5 μL Annexin V-FITC (Shanghai Beyotime Biotechnology Co., Ltd., Shanghai, China) were added. The cells were incubated in the dark for 10 min and washed once with PBS. Then, 400 μL PBS was added to each tube. Apoptosis was detected by flow cytometry, and the apoptosis rate was calculated.

Statistical analyses

Statistical analyses were performed using analysis of variance with SPSS 24.0 software (version 24.0.0; Chicago, IL, United States). Statistical differences were defined as $^aP < 0.05$ and $^bP < 0.01$. The data are presented as the mean ± SD.

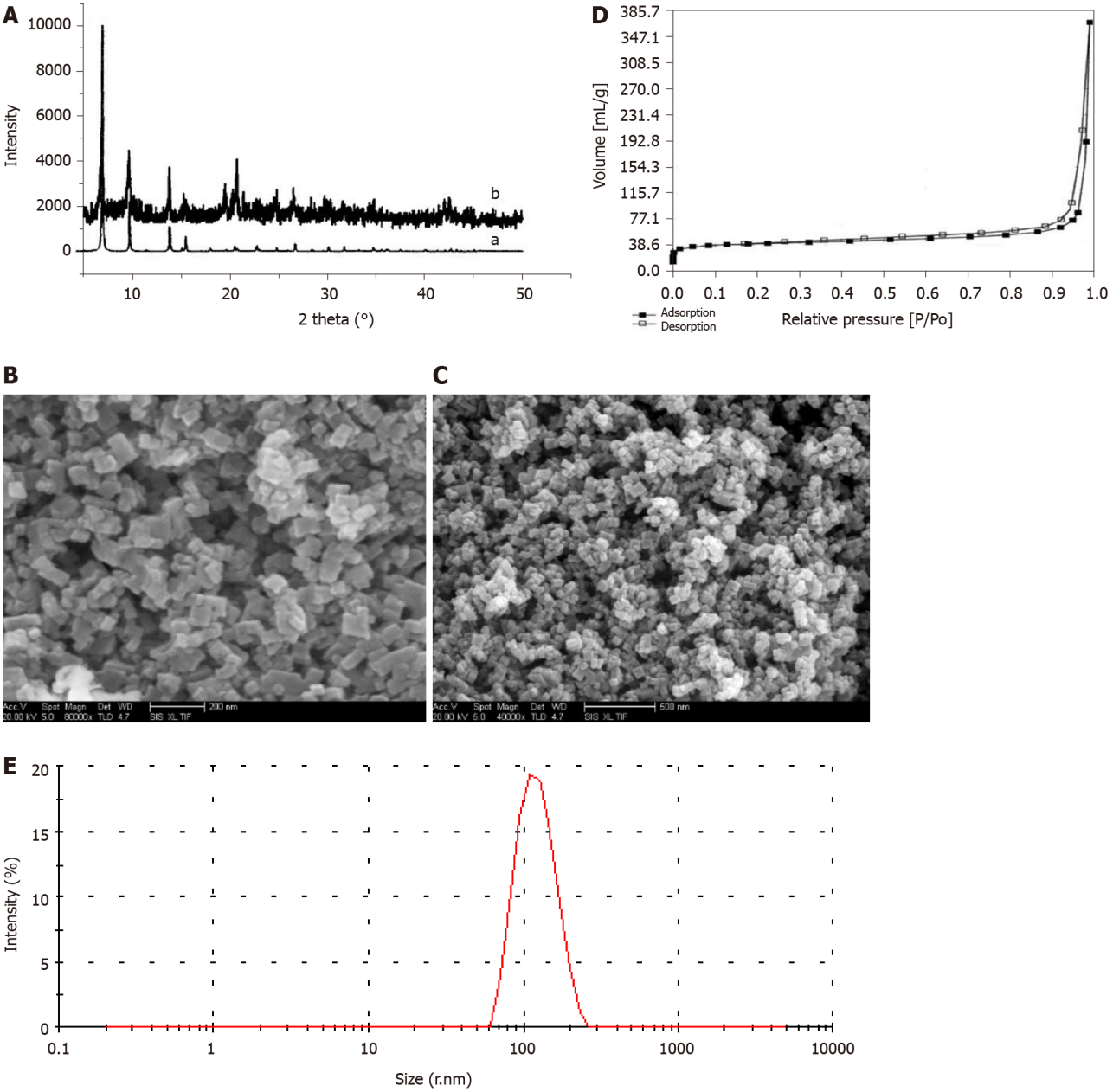


Figure 1 Structure and morphology of IRMOF-3 and norcantharidin-loaded metal-organic framework IRMOF-3 coated with temperature-sensitive gel. A: X-ray diffraction (XRD) spectra of IRMOF-3. Note: XRD patterns of synthetic IRMOF-3 (b) and standard IRMOF-3 (a). XRD patterns of the sample show the same peaks as those of the standard, confirming the high purity of IRMOF-3. The peak patterns of the sample account for the rough appearance of the nanosized particles; B: Scanning electron microscopy (SEM) images of the morphology of IRMOF-3. SEM images of the morphology of IRMOF-3. The particles show a regular square, uniform distribution and a size of 50-100 nm. The single-particle surface is rough, indicating the existence of pores; C: SEM images of the morphology of norcantharidin (NCTD)-loaded metal-organic framework IRMOF-3 coated with temperature-sensitive gel (NCTD-IRMOF-3-Gel). SEM images of the morphology of NCTD-IRMOF-3-Gel showing the same size as that of IRMOF-3; D: Nitrogen adsorption–desorption isotherms of IRMOF-3. Nitrogen adsorption–desorption isotherms. A hysteresis loop phenomenon appears under relatively high pressure, indicating the existence of channels in the sample; E: Particle size distribution of NCTD-IRMOF-3-Gel. The particle size distribution of NCTD-IRMOF-3-Gel indicated that the average particle size was 100 nm.

RESULTS

Structure and morphology

The PXRD patterns shown in Figure 1A illustrate that the IRMOF-3 materials possessed three well-resolved peaks, similar to the standard patterns. The SEM images of IRMOF-3 and NCTD-IRMOF-3-Gel are presented in Figure 1B and Figure 1C which show that the sample consisted of square particles (50-100 nm). The single-particle surface was rough, indicating the existence of pores. SEM images show that the morphology of NCTD-IRMOF-3-Gel had similar size features as those of IRMOF-3. The nitrogen gas (N_2) adsorption–desorption isotherms of IRMOF-3 are shown in Figure 1D. The hysteresis loop phenomenon appeared in the range of relatively high

pressure, indicating the existence of channels in the sample (Figure 1D). The particle size distribution of NCTD-IRMOF-3-Gel indicated that the average particle size was 100 nm (Figure 1E).

NCTD release assay

In the simulated pH 5.0 environment of tumor cells, the drug release data were fitted by a zero-order dynamics equation, first-order kinetic equation, Higuchi equation and Weibull model. The NCTD regression equation obtained is shown in Table 1. The resulting correlation coefficients of the drug release kinetics show that the drug release conforms to the Weibull equation, and the R^2 value is 0.9508.

As shown in Figure 2, the release of NCTD is very fast and is completely finished at 5 h. However, the drug release of NCTD-IRMOF-3 was slower than that of NCTD. The first half of the drug release curve of NCTD was steep and showed a sudden release within 0.6 h. The reason was that the free drug molecules adsorbed on the surface of the nanoparticles diffused rapidly into the medium. Then the curve of NCTD-IRMOF-3 showed a steady slowly release process because the drug in the pores was slowly released. Approximately 5 h were necessary for 50% NCTD release from NCTD-IRMOF-3. After 36 h, the release rate was more than 70%, and the release was basically complete. The release rate of NCTD-IRMOF-3-Gel nanoparticles at 0.3 h was lower than that of the other formulations because NCTD was released gradually with the slow dissolution of poloxamer. After 10 h, the release rate was approximately 50%; after 36 h, the release rate was more than 65%.

In vitro examination

MTT assay: In contrast to that of NCTD- and NCTD-IRMOF-3-treated cells, the inhibition of Hepa1-6 cells treated with NCTD-IRMOF-3-Gel increased in a dose-dependent manner (Figure 3). When the half-maximal inhibitory concentration (IC_{50}) values of each group at different time periods were compared, it was found that these values were 30.59 μg/mL, 93.74 μg/mL, and 112.3 μg/mL with NCTD, NCTD-IRMOF-3, and NCTD-IRMOF-3-Gel, respectively. The inhibitory effects of NCTD-IRMOF-3-Gel on Hepa1-6 cells were stronger than those of NCTD and NCTD-IRMOF-3 and showed a certain sustained-release effect.

Flow cytometry assay of the effects on the Hepa1-6 cell cycle: Figure 4 and Table 2 show that the percentage of the total number of cells in S phase and G2/M phase increased significantly with increasing NCTD-IRMOF-3-Gel concentration, while the proportion of cells in G0/G1 phase decreased significantly. This result indicates that NCTD-IRMOF-3-Gel can block the cell cycle in the S and G2/M phases, and thermosensitive gel nanoparticles may inhibit cell proliferation by inducing cell cycle arrest.

Apoptosis experiment

It can be seen from the figure that the apoptosis rates of NCTD-IRMOF-3-Gel at the high concentration (C) and low concentration (F) were 32.11 μg/mL and 65.60 μg/mL, respectively. Compared with that in the NCTD control group, the apoptosis rate in the NCTD-IRMOF-3-Gel group was highest, which indicated that NCTD-IRMOF-3-Gel could induce the apoptosis of Hepa1-6 cells (Figures 5 and 6).

DISCUSSION

In recent years, with the wide application of medical polymer materials and increased clinical utilization[27,28], research on sustained-release and controlled-release preparations has increased and has become an important research direction. As a new type of drug formulation, sustained- and controlled-release preparations can increase efficacies and reduce side effects compared with traditional drugs. The thermosensitive gel has a hydrophilic three-dimensional network structure, which can be loaded in the liquid state to control drug release. In addition, thermosensitive gel has a stronger affinity, longer retention time and less stimulation in medically relevant locations than traditional gel, especially in mucosal tissue. This type of gel is suitable for all kinds of drug carriers and has now become a research hotspot in pharmaceutics [29,30].

The aim of this project was to prepare NCTD-IRMOF-3-Gel by using the porous material metal-organic framework IRMOF-3 as a drug carrier and NCTD as a model drug. Loading the drug and drug carrier within the thermosensitive gel not only delayed the action time of the drug but also compensated for sudden drug release

Table 1 Release equations and correlation coefficients of norcantharidin-loaded metal-organic framework IRMOF-3 coated with temperature-sensitive gel

	Model	Equation	R^2
DM	Zero-order processes	Q = 1.2786 t + 36.322	0.5383
	First-order processes	In (100-Q) = -0.0268 t + 4.1375	0.667
	Higuchi	$Q = 9.6078\ t^{0.5} + 25.29$	0.7719
	Weibull	InIn (1/1-Q) = 0.3813 Int - 0.9163	0.9508

Table 2 Effects of norcantharidin-loaded metal-organic framework IRMOF-3 coated with temperature-sensitive gel on the cell cycle (*n* = 3)

Groups (μg/mL)	G0/G1	S	G2/M
Blank group	88.1 ± 2.8	7.2 ± 1.9	4.7 ± 2.1
10	21.9 ± 1.6[b]	59.4 ± 2.2[b]	18.7 ± 1.8[a]
20	16.6 ± 1.2[b]	54.1 ± 2.4[b]	29.2 ± 1.9[a]
40	18.3 ± 2.4[b]	45.8 ± 3.1[b]	35.8 ± 2.7[a]

[a]$P < 0.05$.
[b]$P < 0.01$.

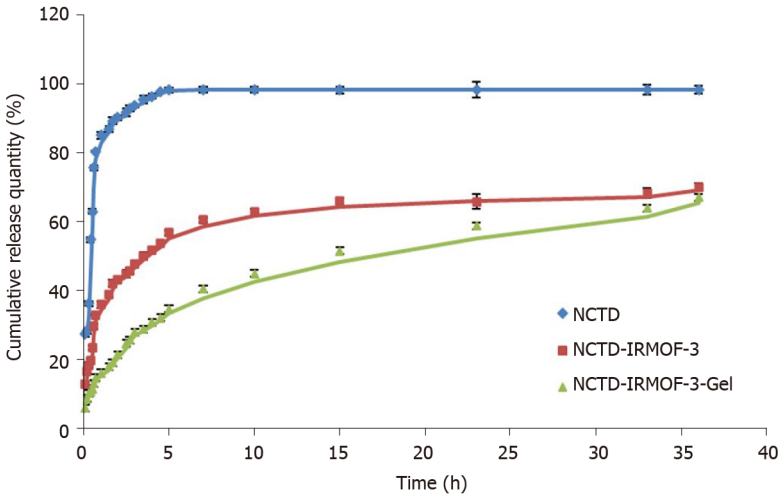


Figure 2 *In vitro* release curves of norcantharidin (NCTD) (blue line), NCTD-loaded metal-organic framework IRMOF-3 (red line), and NCTD-loaded metal-organic framework IRMOF-3 coated with temperature-sensitive gel (green line). After approximately 5 h, 90% of norcantharidin (NCTD) was found in the release medium, while only 50% of NCTD was released from NCTD-loaded metal-organic framework IRMOF-3 (NCTD-IRMOF-3), and 30% of NCTD was released from NCTD-loaded metal-organic framework IRMOF-3 coated with temperature-sensitive gel (NCTD-IRMOF-3-Gel). Every trial was repeated three times. All values are shown as the mean ± SD.

from the metal-organic framework carrier. The XRD pattern of the IRMOFs is consistent with that of the standard materials and the peak pattern is rough, which suggest that IRMOFs are nanoparticles with rough surfaces and high purity. The SEM images of the morphology of IRMOF-3 and NCTD-IRMOF-3-Gel showed that the IRMOFs were square and regular nanoparticles containing pores. NCTD-IRMOF-3-Gel was prepared using a poloxamer thermosensitive gel as the carrier, and its morphology did not change. This result shows that the thermosensitive gel has no effect on the original metal-organic framework structure, solves the problem of sudden drug release, and can reduce the toxicity caused by sudden drug release.

The Brunauer–Emmett–Teller surface areas and micropore volume of NCTD-IRMOF-3-Gel were determined using N_2 adsorption isotherms, which showed that it was a microporous material. The N_2 adsorption method is commonly used to

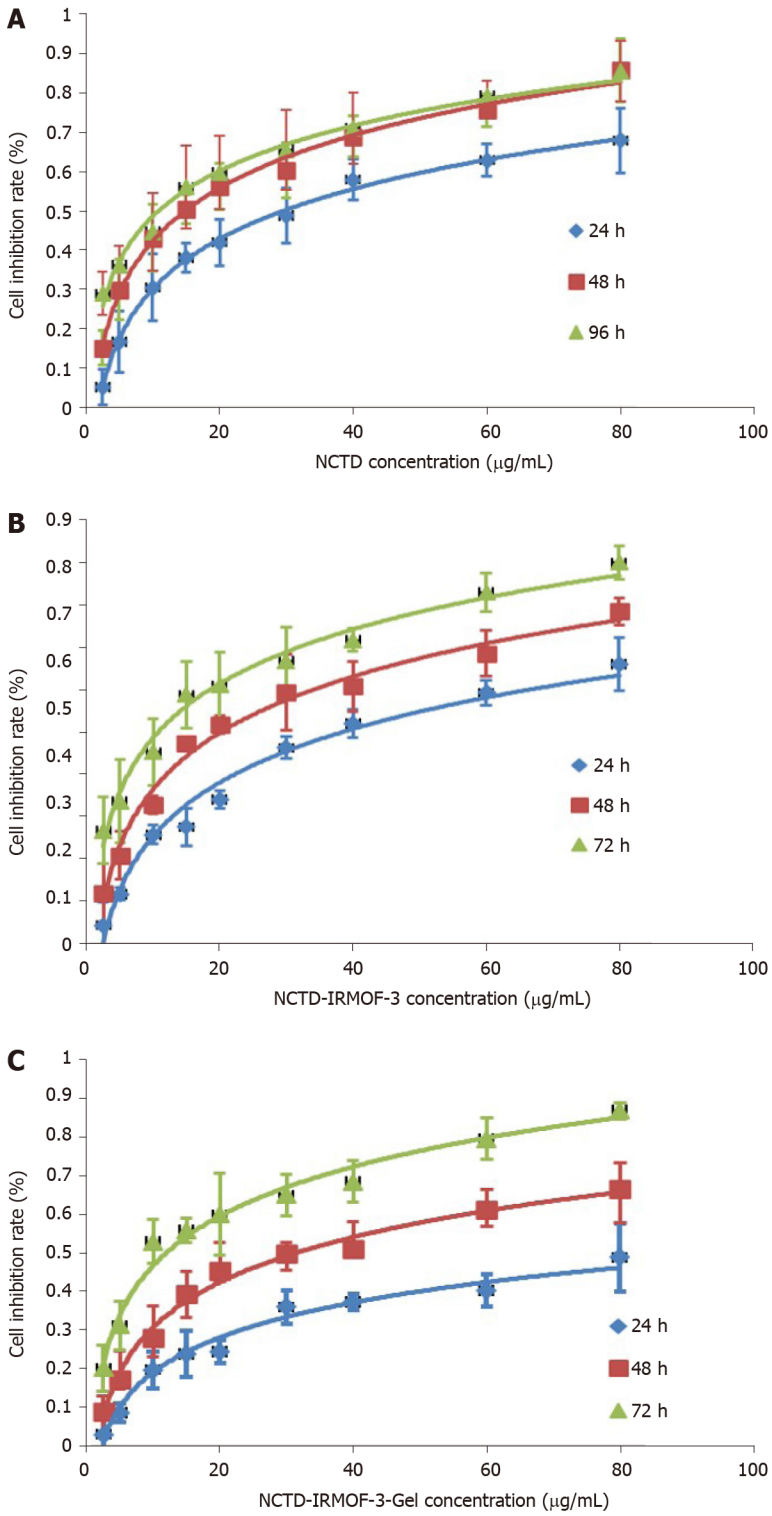


Figure 3 Comparison of the cytotoxicities of norcantharidin (NCTD), NCTD-loaded metal-organic framework IRMOF-3, and NCTD-loaded metal-organic framework IRMOF-3 coated with temperature-sensitive gel. The inhibitory effect of norcantharidin (NCTD)-loaded metal-organic framework IRMOF-3 coated with temperature-sensitive gel (NCTD-IRMOF-3-Gel) (C) on Hepa1-6 cells was stronger than that of NCTD (A) and NCTD-loaded metal-organic framework IRMOF-3 (NCTD-IRMOF-3) (B) and showed a certain sustained-release effect.

determine the specific surface area and pore size of nanomaterials. The determination principle of N_2 adsorption is that the surface pores of porous materials will adsorb nitrogen at liquid nitrogen temperatures. The flat areas in the low-pressure section were caused by the nanopores. The N_2 adsorption quantity increased suddenly, which generated a hysteresis loop, indicating the existence of micropores, and this phenomenon was caused by capillary condensation. After zeta potential analysis of the particle size distribution, NCTD-IRMOF-3-Gel was shown to have a particle size of approximately 100 nm and good dispersibility.

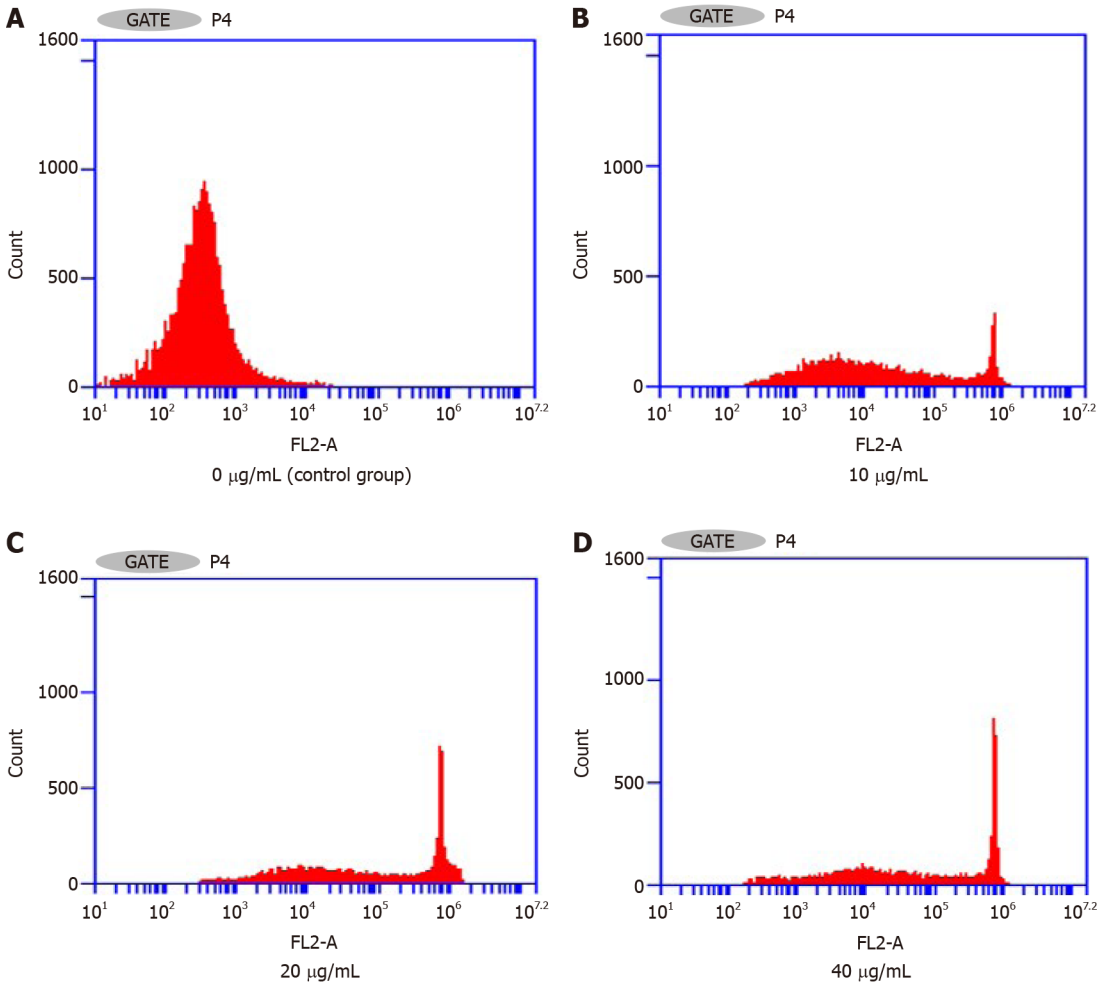


Figure 4 Effects of norcantharidin-loaded metal-organic framework IRMOF-3 coated with temperature-sensitive gel on the cell cycle. The percentage of total cells in S and G2/M phases increased significantly with increasing norcantharidin (NCTD)-loaded metal-organic framework IRMOF-3 coated with temperature-sensitive gel (NCTD-IRMOF-3-Gel) concentration, and the proportion of cells in G0/G1 phase decreased significantly. A: Control group; B: 10 μg/mL; C: 20 μg/mL; D: 40 μg/mL. G1 phase: DNA presynthetic phase, where mitosis is complete before DNA replication begins; G2 phase: DNA synthesis replication phase, where DNA replication is complete before mitosis begins; M phase: Cell division phase; S phase: DNA synthesis replication phase.

The kinetics of *in vitro* drug release can effectively determine the profile of *in vitro* drug release and predict the conditions of *in vivo* drug release. From the drug release curve, NCTD was released quickly, with basically complete release at 5 h. NCTD-IRMOF-3 nanoparticles released NCTD more slowly than free NCTD treatment and showed sudden release within 0.6 h. After 36 h, the release rate was more than 70%, and the release was basically completed. NCTD-IRMOF-3-Gel nanoparticles showed a significantly slower release trend, and the degree of release at 0.3 h was lower than that in the other groups, which was due to the gradual release of NCTD with the slow dissolution of poloxamer. After 36 h, the release rate reached more than 65%. We speculated that NCTD-IRMOF-3-Gel nanoparticles had a certain sustained-release effect and could effectively improve the drug release process.

The MTT assay demonstrated the cytotoxicity of the NCTD-IRMOF-3-Gel nanoparticles. At the same concentration, the inhibition rate of each group of drugs acting on Hepa1-6 cells increased with the extension of time. The inhibition rate of the free drug group was slightly lower than that of the nanoparticle group, but it still had a killing effect on the cells. Compared with that of NCTD-IRMOF-3-Gel, the inhibition rate of NCTD-IRMOF-3 was slightly low, indicating that the thermosensitive gel-coated nanoparticles had a better inhibitory effect on cells. The inhibition rate of the nanoparticle group was low at 24 h and gradually increased after 48 h to the level of inhibition of the free drug, indicating that the drug-loaded nanoparticle-thermosensitive gel group presented an obvious sustained-release effect. Meanwhile, the cell cycle study using flow cytometry showed that NCTD-IRMOF-3-Gel could block the S phase and G2/M phase of the cell cycle, and thermosensitive gel-suspended nanoparticles may inhibit cell proliferation by blocking the cell cycle. The apoptosis

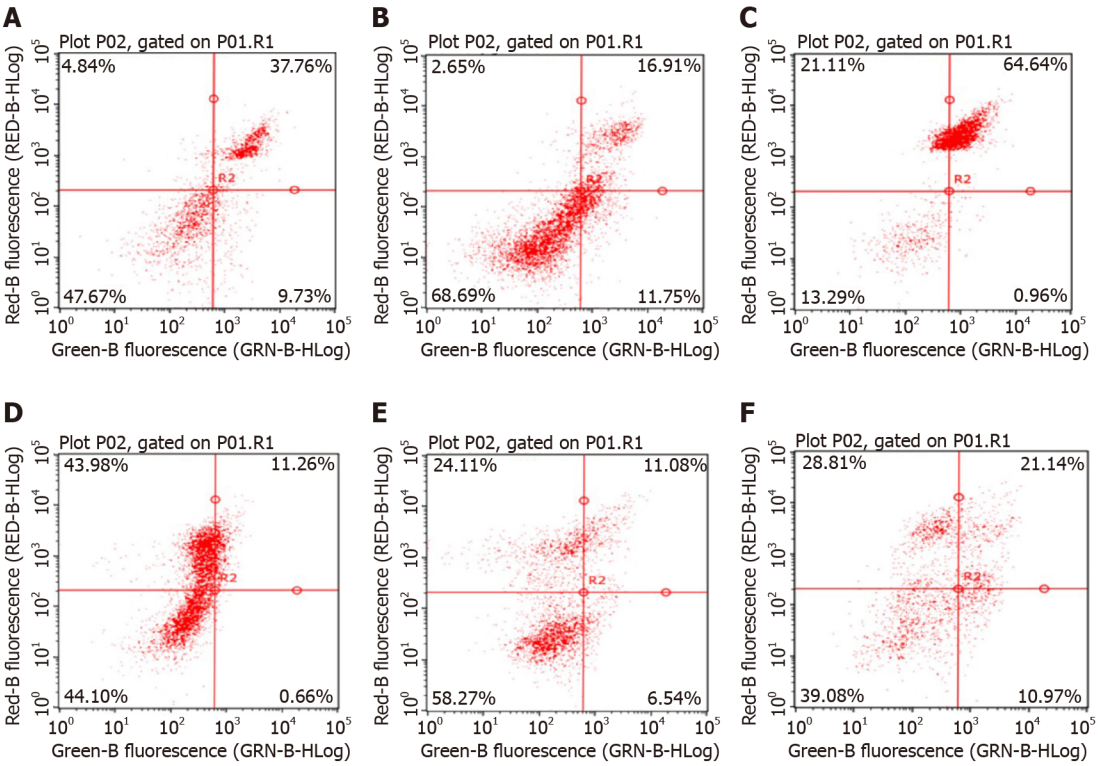


Figure 5 Apoptosis rates of Hepa1-6 cells after 48 h. Apoptosis rates of norcantharidin (NCTD)-loaded metal-organic framework IRMOF-3 coated with temperature-sensitive gel (NCTD-IRMOF-3-Gel) at the high concentration (C) and low concentration (F) were 32.11 μg/mL and 65.60 μg/mL, respectively. Compared with the NCTD control group, the apoptosis rate in the NCTD-IRMOF-3-Gel group was highest, which indicated that NCTD-IRMOF-3-Gel could induce the apoptosis of Hepa1-6 cells. A: 50 μg/mL NCTD-IRMOF-3; B: 50 μg/mL NCTD; C: 50 μg/mL NCTD-IRMOF-3-Gel; D: 25 μg/mL NCTD-IRMOF-3; E: 25 μg/mL NCTD; F: 25 μg/mL NCTD-IRMOF-3-Gel.

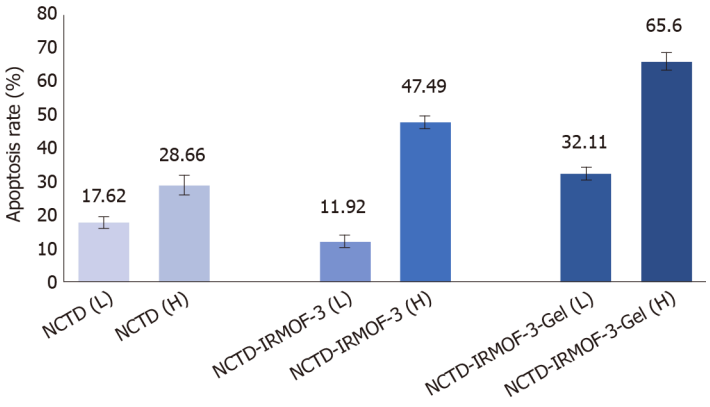


Figure 6 Apoptosis rates of norcantharidin (NCTD), NCTD-loaded metal-organic framework IRMOF-3, and NCTD-loaded metal-organic framework IRMOF-3 coated with temperature-sensitive gel. Compared with the norcantharidin (NCTD) control group, the apoptosis rate in the NCTD-loaded metal-organic framework IRMOF-3 coated with temperature-sensitive gel (NCTD-IRMOF-3-Gel) group was highest, which indicated that NCTD-IRMOF-3-Gel can induce the apoptosis of Hepa1-6 cells.

rates of NCTD-IRMOF-3-Gel at high concentrations and low concentrations were determined, which indicated that NCTD-IRMOF-3-Gel could induce the apoptosis of Hepa1-6 cells.

CONCLUSION

Based on the results of this study, NCTD-loaded IRMOF-3 nanoparticles incorporated into a thermosensitive gel appeared to be a useful tool for cancer treatment because of the enhanced inhibition rate of cancer cells and controlled release of drugs from these

nanocarriers. Our future studies will focus on elucidating the activity of the drug delivery system and its effects on the mechanism of action of the encapsulated anticancer drug.

ARTICLE HIGHLIGHTS

Research background

Norcantharidin (NCTD) is suitable for the treatment of primary liver cancer, especially early and middle primary liver cancer. As a new type of drug formulation, sustained- and controlled-release preparations can increase the efficacy and reduce the side effects compared with traditional drugs. Metal-organic frameworks (MOFs) have potential applications in drug carriers. The thermosensitive gel has a hydrophilic three-dimensional network structure, which can be loaded in the liquid state to control drug release.

Research motivation

The side effects of NCTD have limited its application in liver cancer, which has prompted the development of sustained- and controlled-release preparations.

Research objectives

This study established a liver-targeting therapy in which NCTD is loaded into IRMOF-3 coated with a thermosensitive gel (NCTD-IRMOF-3-Gel), which can be efficiently delivered to liver cancer cells and slowly released.

Research methods

NCTD-loaded IRMOF-3 coated with a temperature-sensitive gel (NCTD-IRMOF-3-Gel) was obtained by a coordination reaction. The apparent characteristics and *in vitro* release of NCTD-IRMOF-3-Gel were investigated. Cell cytotoxicity assays, flow cytometry and apoptosis experiments on mouse hepatoma (Hepa1-6.) cells were used to determine the anti-liver cancer activity of NCTD-IRMOF-3-Gel in *in vitro* models.

Research results

The particle size of NCTD-IRMOF-3-Gel was 50-100 nm, and the particle size distribution was uniform. The release curve showed that NCTD-IRMOF-3-Gel had an obvious sustained-release effect. The cytotoxicity assays showed that the free drug NCTD and NCTD-IRMOF-3-Gel treatments markedly inhibited Hepa1-6 cell proliferation, and with increasing drug concentrations, the inhibition rate increased. By flow cytometry, NCTD-IRMOF-3-Gel was observed to block the Hepa1-6 cell cycle in the S and G2/M phases, and the thermosensitive gel nanoparticles may inhibit cell proliferation by inducing cell cycle arrest. Apoptosis experiments showed that NCTD-IRMOF-3-Gel induced the apoptosis of Hepa1-6 cells.

Research conclusions

NCTD-loaded IRMOF-3 nanoparticles incorporated into a thermosensitive gel appeared to be a useful tool for cancer treatment because of the enhanced inhibition rate of cancer cells and controlled release of drugs from these nanocarriers.

Research perspectives

Thermosensitive gel-encapsulated IRMOF-3 has great advantages as an antitumor drug carrier and provides some ideas for passive targeting therapy of tumors.

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ORIGINAL ARTICLE

Basic Study

Ubiquitin-specific protease 15 contributes to gastric cancer progression by regulating the Wnt/β-catenin signaling pathway

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Author contributions: Zhong M, Zhou L, and Fang Z contributed equally to this work; Zhong M performed data acquisition and drafted the manuscript; Zhou L and Fang Z completed the statistical analyses; Yao YY and Zou JP were involved in critically revising the manuscript for important intellectual content; Deng J, Xiong JP, and Xiang XJ conceived and designed the study.

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Abstract

BACKGROUND

Ubiquitin-specific protease 15 (USP15) is an important member of the ubiquitin-specific protease family, the largest deubiquitinase subfamily, whose expression is dysregulated in many types of cancer. However, the biological function and the underlying mechanisms of USP15 in gastric cancer (GC) progression have not been elucidated.

AIM

To explore the biological role and underlying mechanisms of USP15 in GC progression.

METHODS

Bioinformatics databases and western blot analysis were utilized to determine the expression of USP15 in GC. Immunohistochemistry was performed to evaluate the correlation between USP15 expression and clinicopathological characteristics of patients with GC. A loss- and gain-of-function experiment was used to investigate the biological effects of USP15 on GC carcinogenesis. RNA sequencing, immunofluorescence, and western blotting were performed to explore the potential mechanism by which USP15 exerts its oncogenic functions.

RESULTS

USP15 was up-regulated in GC tissue and cell lines. The expression level of USP15 was positively correlated with clinical characteristics (tumor size, depth of invasion, lymph node involvement, tumor-node-metastasis stage, perineural invasion, and vascular invasion), and was related to poor prognosis. USP15

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Data sharing statement: No additional data are available.

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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knockdown significantly inhibited cell proliferation, invasion and epithelial-mesenchymal transition (EMT) of GC *in vitro*, while overexpression of USP15 promoted these processes. Knockdown of USP15 inhibited tumor growth *in vivo*. Mechanistically, RNA sequencing analysis showed that USP15 regulated the Wnt signaling pathway in GC. Western blotting confirmed that USP15 silencing led to significant down-regulation of β-catenin and Wnt/β-catenin downstream genes (c-myc and cyclin D1), while overexpression of USP15 yielded an opposite result and USP15 mutation had no change. Immunofluorescence indicated that USP15 promoted nuclear translocation of β-catenin, suggesting activation of the Wnt/β-catenin signaling pathway, which may be the critical mechanism promoting GC progression. Finally, rescue experiments showed that the effect of USP15 on gastric cancer progression was dependent on Wnt/β-catenin pathway.

CONCLUSION

USP15 promotes cell proliferation, invasion and EMT progression of GC *via* regulating the Wnt/β-catenin pathway, which suggests that USP15 is a novel potential therapeutic target for GC.

Key Words: Ubiquitin-specific protease 15; Gastric cancer; Wnt/β-catenin; Cell proliferation; Cell invasion; Epithelial-mesenchymal transition

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Core Tip: Ubiquitin-specific protease 15 (USP15) was upregulated in gastric cancer (GC) cells and tissues, and was associated with a poor prognosis in patients with GC. USP15 promoted cell proliferation, invasion, and epithelial-mesenchymal transition of GC cells *in vitro* and tumor growth *in vivo*. Mechanistic studies showed that USP15 functioned as a tumor promoter in GC by regulating the Wnt/β-catenin signaling pathway. Thus, USP15 is expected to be a novel potential target for GC therapy.

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INTRODUCTION

Gastric cancer (GC) has a high incidence worldwide and is one of the main causes of cancer-related deaths, especially in China[1,2]. Although there have been great advances in surgical procedures and targeted chemotherapy in recent years, the results are still not satisfactory and the survival rate is low, with median overall survival (OS) less than 12 mo[3-5]. Therefore, identifying novel potential targets for GC diagnosis and therapy and elucidating the underlying mechanisms of disease progression are essential for the prevention and treatment of GC.

In recent years, increasing evidence has shown that ubiquitin-specific proteases (USPs), the largest deubiquitinase subfamily, plays an important role in GC. For example, USP14[6], USP42[7], and USP44[8] are upregulated in GC and can be used as independent prognostic markers in GC patients. USP15, one of the most important members of the USP family, has been found to have some amplifications in many tumors. The N terminus of the protein encoding USP15 includes a ubiquitin-specific protease (DUSP) domain and two ubiquitin-like (UBL) domains, which can specifically remove the substrate protein by monoubiquitination and polyubiquitination modification[9]. The active site of the USP15 protein is located at Cys-269, and mutation of Cys269 to Ser (USP15 C269S) can inhibit enzyme activity[10]. Previous studies have reported that USP15 is upregulated in the liver and pancreatic cancer, and is associated with poor prognosis[11,12]. Mechanistically, USP15 can activate the transforming growth factor β (TGF-β) signaling pathway and promote the progression of advanced malignant glioma by combining the SMAD-specific E3 ubiquitin protein ligase 2

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complex and deubiquitinating and thus stabilizing the TGF b type I receptor[13]. In addition, USP15 can negatively regulate the function of p53 through affecting de-ubiquitination and stabilizing MDM2. Interestingly, inhibiting the activity of USP15 can induce tumor apoptosis and improve the antitumor T-cell response[14]. However, the role of USP15 in GC and its potential mechanisms have not been identified.

The Wnt/β-catenin signaling pathway is involved in many cellular processes such as tumor growth, differentiation and invasion, and tumorigenesis[15]. It is often activated in many types of cancer, and the nuclear accumulation of β-catenin is an important sign of Wnt signaling activation[16]. The activation of β-catenin can activate many oncogenes including c-myc and cyclin D1, and regulate cell proliferation, cell cycle progression and apoptosis during tumorigenesis[17-19]. However, the mechanisms of Wnt/β-catenin activation in GC have not been fully elucidated.

We found that USP15 was upregulated in GC cells and tissues, and was associated with a poor prognosis in GC patients. USP15 promoted cell proliferation, invasion, and epithelial-mesenchymal transition (EMT) of GC cells *in vitro* and tumor growth *in vivo*. Mechanistic studies showed that USP15 functioned as a tumor promoter in GC by regulating the Wnt/β-catenin signaling pathway. Thus, USP15 is expected to be a novel potential target for GC therapy.

MATERIALS AND METHODS

Tissue samples

Paraffin-embedded GC samples, including cancerous tissues (n = 115) and adjacent tissues (n = 30), from May 2011 and May 2013, were obtained from the First Affiliated Hospital of Nanchang University (Nanchang, China). The clinicopathological characteristics of these patients are shown in Table 1. The fresh GC tissues (n = 8) and corresponding adjacent noncancerous tissues were stored in liquid nitrogen until use. This study obtained ethical approval from the Human Research Ethics Committee of the First Affiliated Hospital of Nanchang University.

Cell lines and culture

Human GC cell lines (SGC-7901, HGC-27, MKN-45, MGC-803, BGC-823, and AGS) and the human immortalized gastric epithelial cell line (GES-1) were purchased from the Beijing Beina Chuanglian Institute of Biotechnology (Beijing, China). The cells were cultured in (RPMI-1640) or Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS; HyClone, Logan, UT, United States) in an incubator with 5% CO_2 at 37 °C.

Immunohistochemistry

Immunostaining of USP15 proteins in 115 clinical GC samples followed previously described methods[20]. A primary antibody against USP15 (1:100, #66310; Cell Signaling Technology, Danvers, MA, United States) was used to detect the expression of USP15. All staining scores were evaluated blindly by two pathologists based on staining intensity and positive staining ratio. The grading standard of immunohistochemistry was carried out as previously described [20].

Cell Counting Kit-8 assay and colony formation assay

At 48 h after transfection, 2000 GC cells per well were seeded into a 96-well plate for the Cell Counting Kit-8 (CCK-8) assay and 1000 GC cells per well were seeded into a 6-well plate for the colony formation assay as previously described[21].

Wound healing assay

At 48 h after transfection, 5 × 10^5 GC cells per well were seeded into a 6-well plate, and the cells were starved for 24 h until complete fusion. Straight lines were drawn with a sterile 10-μL pipette tip to form wounds. Then the cells were carefully washed with phosphate-buffered saline (PBS) and cultured in serum-free medium. Images were captured at 0, 24, and 48 h to assess wound closure.

Transwell assay

The transwell assay was performed *via* using 8-μm transwell chambers (Merck KGaA, Darmstadt, Germany) with or without 60 μL Matrigel gel (BD Biosciences, Hercules, CA, United States), and then the chambers were put in each well of a 24-well plate. Cells of each group (5 × 10^4) were placed in 200 μL serum-free medium for 48 h after transfection, and subsequently transferred to the upper compartment of the above

Table 1 Ubiquitin-specific protease 15 expression and clinicopathological characteristics of gastric cancer patients

Parameters	*n*	USP15 expression		*P* value
		low	High	
Gender				
Male	62	20	42	0.218
Female	53	23	30	
Age in year				
≤ 60	53	18	35	0.482
> 60	62	25	37	
Differentiation				
Poor	69	25	44	0.753
Moderate/well	46	18	28	
Tumor size in cm				
≤ 4	66	32	34	0.004
> 4	49	11	38	
TNM stage				
I + II	58	28	30	0.015
III + IV	57	15	42	
Depth of invasion				
T1 + T2	54	27	27	0.009
T3 + T4	61	16	45	
LNI				
N0	43	24	19	0.002
N1 + N2+N3	72	19	53	
Perineural invasion				
No	51	25	26	0.021
Yes	64	18	46	
Vascular invasion				
No	50	27	23	0.001
Yes	65	16	49	
Total	115	43	72	

GC: Gastric cancer; LNI: Lymph node involvement; TNM: Tumor-node-metastasis; USP15: Ubiquitin-specific protease 15.

chambers. The lower chamber contained RPMI-1640 with 10% FBS. After 36 h of incubation, the cells that had migrated or invaded to the bottom side of the chamber were fixed with methanol, and then stained with crystal violet.

Immunofluorescence

We dipped the coverslip into the culture medium to allow the cells to attach and grow, and then washed the cells three times with PBS. At room temperature, the cells were fixed on a coverslip with 4% tetraformaldehyde for 20 min, and then were washed again three times with PBS. After a 10 min incubation with 0.5% Triton X-100, the cells were blocked in 5% bovine serum albumin for 2 h and then were incubated with anti-β-catenin antibody (1:200 dilution; Cell Signaling Technology) at 4 °C. After washing three times with PBS, cells were incubated with secondary antibody (1:50 dilution, ab150077; Abcam, Cambridge, MA, United States) for 1 h at room temperature. The coverslips were subsequently washed three times with PBS and then were stained with 4′,6-diamidino-2-phenylindole (DAPI). Fluorescence images were captured *via*

laser confocal microscopy.

Western blotting

Western blotting was performed following as previously described[22]. The following primary antibodies were used: USP15 (1:2000, #66310; Cell Signaling Technology), E-cadherin (1:1000, ab1416; Abcam), N-cadherin (1:1000, ab18203; Abcam), vimentin (1:1500, ab8978; Abcam), c-Myc (1:1500, #5605; Cell Signaling Technology), β-catenin (1:2000, #8480; Cell Signaling Technology), cyclin D1 (1:1000, #2978; Cell Signaling Technology), and GAPDH (1:2000, #60004-1-Ig; Proteintech, Rosemont, IL, United States).

Plasmid construction and cell transfection

To knock down the expression of USP15, three different small interfering RNAs (siRNAs) and a negative control (NC) were designed as followed: USP15-Homo-249, 5′-GGAACACCUUAUUGAUGAATT-3′; USP15-Homo-1150, 5′-GCAGAUGGAAGGC-CAGAUATT-3′; USP15-HoMo-1382, 5′-CCAAACCUAUGCAGUACAATT-3′; and a NC siRNA, 5′-UUCUCCGAACGUGUCACGUTT-3′. USP15 overexpression plasmid (USP15: NM_006313.2) and USP15 mutated plasmid (USP15-C269S) were based on pcDNA3.1 plasmid. The above siRNA and plasmid were synthesized by GenePharma (Suzhou, China). Cells were grown to 50%–60% confluency and transfected using TurboFect transfection reagent (R0532; Thermo Scientific Scientific, Waltham, MA, United States).

RNA sequencing analysis

The isolated USP15 knockdown and control BGC-823 cells were used for cDNA amplification and RNA sequencing (RNA-seq) library preparation. RNA-seq was performed by Beijing Novel Bioinformatics Co. Ltd. (Beijing, China). Genes with a false discovery rate < 5% and a fold change > 2.0 that met the established threshold criteria were considered to be significantly differentially expressed.

Nude mouse tumor cell xenograft assay

Short hairpin RNAs (shRNAs) targeting USP15 or scramble shRNAs were subcloned into the lentiviral expression vector (Jikai Co. Shanghai, China). BGC□823 cells transfected with LV-shUSP15 or LV-scramble shRNA was stably expressed and screened by puromycin. The stably expressed strain was amplified and inoculated at a rate of 5×10^6 cells per animal into 5- to 6-wk-old BALB/c-nu mice. Tumor volume was measured every 3 d and calculated according to the formula: volume (mm^3) = (length × $width^2$)/2. Mice were sacrificed after 28 d and xenograft tumors were measured and weighed. Proteins were extracted from tumors and USP15 and β-catenin expression was detected by western blotting.

Statistical analyses

We statistically analyzed the data using SPSS version 26.0 software (Chicago, IL, United States). The relationship between clinical characteristics and USP15 expression was evaluated by the χ^2 test. The Kaplan–Meier method was performed to determine the OS curve of all enrolled GC patients. Student's *t*-test was used to determine the mean difference between two groups. $P < 0.05$ was considered statistically significant.

RESULTS

USP15 is upregulated in GC cells and tissues and is associated with a poor prognosis in patients with GC

First, an online database, cBioPortal for Cancer Genomics (http://www.cbioportal.org/)[23], showed that USP15 was amplified in many types of tumor including GC (Figure 1A). TIMER (https://cistrome.shinyapps.io/timer/)[24] and UALCAN database (http://ualcan.path.uab.edu/)[25], based on The Cancer Genome Atlas (TCGA) database, indicated that the mRNA levels of USP15 in GC tissues were higher than those in normal tissues (Figure 1B and C). To confirm the protein expression level of USP15 in GC, western blotting was conducted on GC cell lines and tissues. Most GC cell lines expressed a higher level of USP15 than the gastric epithelial cell line GES-1 (Figure 1D). USP15 was elevated in most of the eight pairs of clinical GC tissues and their adjacent normal tissues (Figure 1E).

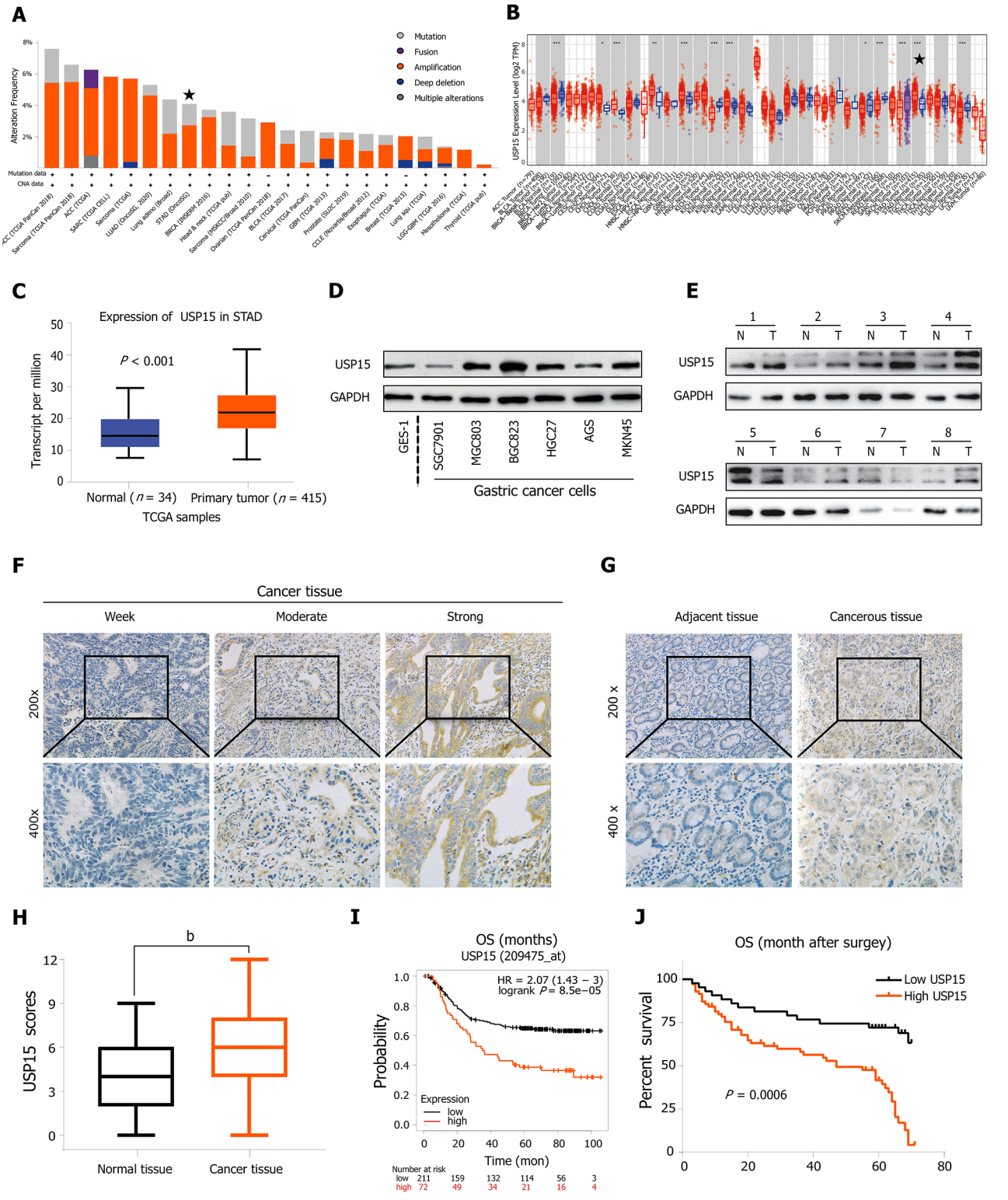


Figure 1 Ubiquitin-specific protease 15 is upregulated in gastric cancer cells and tissues, and is associated with a poor prognosis in gastric cancer patients. A: Frequency of ubiquitin-specific protease 15 (USP15) in various cancers, including gastric cancer (GC). The data were derived from the cBioPortal for Cancer Genomics; B, C: USP15 mRNA expression level of cancer tissue and normal tissue in GC. Analysis of The Cancer Genome Atlas data from TIMER and UALCAN databases; D: Protein expression of USP15 in GES-1 cell line and GC cell lines; E: Protein expression of USP15 in GC tissue and adjacent noncancerous tissues (n = 8); F, G: Representative images of immunohistochemical staining of USP15 in human GC tissues and adjacent noncancerous tissues; H: USP15 staining scores of GC and normal tissues; I: Correlation between USP15 expression level and overall survival (OS) in GC patients obtained from Kaplan–Meier Plotter databases (209475_at, HR = 2.07 (1.43-3), log rank $P = 8.5e^{-05}$); J: Kaplan–Meier curve stratified by USP15 expression in 115 GC patients (log-rank test, P = 0.0006). $^{b}P < 0.01$, data were expressed as the mean ± standard error of the mean.

As USP15 was found to be upregulated in GC, we confirmed its clinical significance *via* using immunohistochemistry. The staining of USP15 protein ranged from weak to strong and located in the cytoplasm (Figure 1F), which showed that USP15 was markedly increased in GC tissue sections, whereas USP15 staining was weak or negative in noncancerous tissue sections (Figure 1G). The staining scores of USP15 in adjacent tissues were significantly lower than those in GC tissues, which were considered significantly different (Figure 1H).

Subsequently, we evaluated the correlation between the staining score of USP15 and the clinicopathological characteristics of patients. There was no significant difference among patient gender, age, differentiation, and USP15 expression; however, tumor size (P = 0.004), tumor-node-metastasis (TNM) stage (P = 0.015), depth of invasion (P = 0.009), lymph node involvement (LNI) (P = 0.002), perineural invasion (P = 0.021), and vascular invasion (P = 0.001) were significantly associated with USP15 expression in GC. Consistent with the results obtained from Kaplan–Meier Plotter Database Analysis[26] (http://kmplot.com/analysis/) , the Kaplan–Meier curve stratified by USP15 expression in these 115 GC patients showed that patients with lower USP15 expression had longer OS (Figure 1I and J).

Knockdown of USP15 inhibits cell proliferation, invasion, and EMT progression of GC in vitro

As shown above, BGC-823 and MKN-45 cells had high expression of USP15. siRNA-mediated knockdown of USP15 expression in BGC-823 and MKN-45 cells was used to detect the function of USP15 *in vitro*. Western blotting was used to confirm the silencing efficiency of USP15 in GC cells (Figure 2A). The results of CCK-8 and colony formation assays showed that the proliferation rate and colony formation ability were markedly decreased in the USP15-siRNA-1/2 group compared to the NC group (Figure 2B and C). Based on correlation of USP15 expression and lymph node status, perineural and vascular invasion, wound healing and transwell assays were used to evaluate the role of USP15 in tumor cell migration and invasion. As shown in Figure 2D and Figure 2E, USP15 silencing suppressed GC cell migration and invasion. In addition, knockdown of USP15 upregulated E-cadherin and downregulated N-cadherin and vimentin (Figure 2F).

USP15 overexpression promotes cell proliferation, invasion, and EMT progression in GC

We explored the cellular behavioral changes caused by overexpression of USP15. A stably transfected cell line with USP15 overexpression plasmid, USP15 mutant plasmid (USP15-C269S), and a NC (empty-vector) cell line were established in SGC7901 cells. Western blotting confirmed the transfection efficiency (Figure 3A). Compared with the empty vector group, proliferation of the USP15 group was significantly enhanced, while the USP15-C269S group had no changes (Figure 3B and C). Overexpression of USP15 promoted GC cell migration and invasion, while USP15-C269S did not (Figure 3D and E). Western blotting analysis showed that overexpression of USP15 upregulated vimentin and N-cadherin but downregulated E-cadherin (Figure 3F). Collectively, these data demonstrated that USP15 overexpression promoted GC proliferation, invasion, and EMT progression.

USP15 regulates the Wnt/β-catenin pathway in GC cells

To explore the potential molecular mechanism responsible for the effects of USP15 on GC progression, the whole transcriptome profiles of BGC-823 cells with USP15 knockdown or NC were analyzed by RNA-seq. The transfection efficiency was confirmed by western blotting (Figure 4A). The most differentially expressed genes (DEGs) (29829) were displayed on the heat map (Figure 4B). Among the 2343 significant DEGs (adjusted $P < 0.05$), transcripts of 1134 genes were upregulated and transcripts of 1209 were downregulated in USP15 knockdown groups compared to the control groups (Figure 4C). Gene Ontology enrichment analyses showed that the difference in Wnt signaling pathway was the most obvious (Figure 4D) among the enriched pathways.

As one of the most classic Wnt signaling pathways, the Wnt/β-catenin pathway has been involved in multiple physiological processes of GC progression. To confirm the role of the Wnt/β-catenin signaling pathway in the malignant biological behavior in GC mediated by USP15, western blotting was performed to investigate expression of β-catenin and Wnt/β-catenin downstream genes (including c-myc and cyclin D1). USP15 knockdown resulted in downregulation of the protein level of β-catenin, c-Myc and cyclin D1, while USP15 overexpression yielded opposite results and there was no

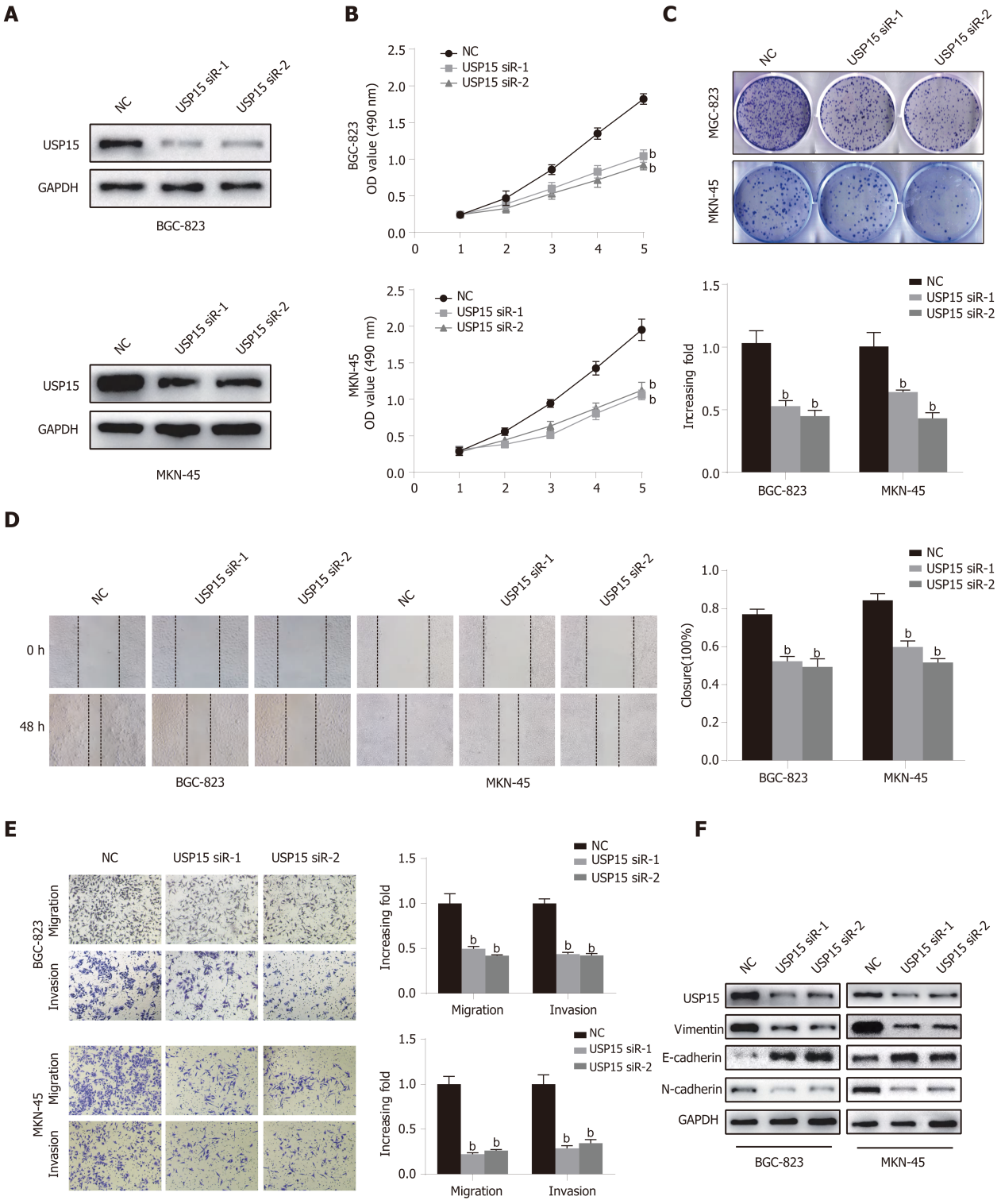


Figure 2 Knockdown of ubiquitin-specific protease 15 inhibits cell proliferation, invasion, and epithelial-mesenchymal transition progression of gastric cancer *in vitro*. A: BGC-823 and MKN-45 cells were transfected with ubiquitin-specific protease 15 (USP15) small interfering RNA or negative control for 48 h, and the efficiency was detected by Western blotting; B and C: Cell Counting Kit-8 assay and colony formation assay evaluated cell proliferation ability; D and E: Wound healing assay and transwell assays evaluated migration and invasion; F: Western blotting detected epithelial-mesenchymal transition markers (E-cadherin, vimentin, and N-cadherin). $^{b}P < 0.01$, data were expressed as mean ± standard error of the mean.

change in USP15 C269S group (Figure 4E). In addition, immunofluorescence assay showed that USP15 knockdown significantly reduced nuclear β-catenin accumulation compared with the control groups, while USP15 overexpression yielded opposite results, and there was no change in the USP15 C269S group (Figure 4F).

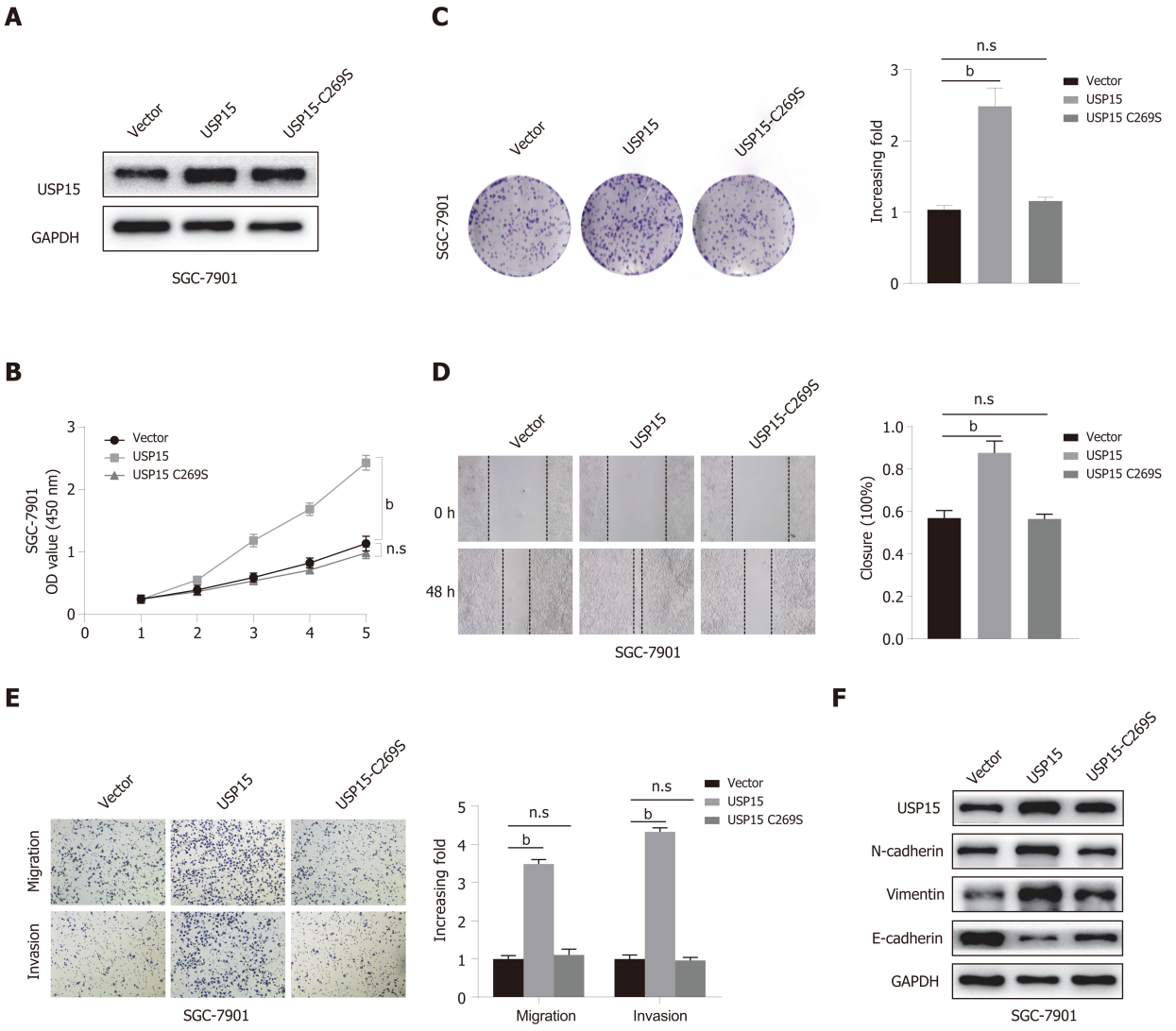


Figure 3 Overexpression of ubiquitin-specific protease 15 promotes proliferation, invasion, and epithelial–mesenchymal transition of gastric cancer cells. A: SGC-7901 cells were transfected with ubiquitin-specific protease 15 (USP15) overexpression plasmid, USP15-C269S plasmid (USP15 mutant) or empty vector for 48 h, and the efficiency was detected by Western blotting; B, C: Cell Counting Kit-8 assay and colony formation assay evaluated cell proliferation ability; D, E: Wound healing assay and transwell assay evaluated migration and invasion; F: Western blotting detected epithelial–mesenchymal transition markers (E-cadherin, vimentin, and N-cadherin). $^{b}P < 0.01$, data are expressed as the mean ± standard error of the mean. n.s: Not significant.

Lithium chloride partly reversed the effects of USP15 knockdown on GC progression

To further clarify whether the function of USP15 in GC was mediated by the Wnt/β-catenin pathway, we performed a rescue experiment using lithium chloride (LiCl) (Wnt/β-catenin pathway activator). The cell proliferation ability of BGC-823 and MKN-45 cells transfected with USP15 siRNA-1 was significantly elevated after treatment with LiCl compared to the untreated group (Figure 5A and B). Furthermore, the inhibition of invasion by USP15 knockdown can also be partly reversed by LiCl (Figure 5C). In addition, LiCl-treatment induced upregulation of β-catenin, c-myc, and cyclin D1 (Figure 5D). The above findings suggest that the function of USP15 on GC progression is dependent on Wnt/β-catenin pathway.

USP15 knockdown inhibits tumor growth in vivo

We investigated the function of USP15 *in vivo*. BGC-823 cells transfected with LV-shUSP15 or LV-scramble shRNA were subcutaneously injected into nude mice to establish a xenograft mouse model. After the mice were sacrificed on day 29, we obtained tumor images (Figure 6A). Compared with the scramble shRNA group, USP15 knockdown reduced tumor volume and weight (Figure 6B and C). In addition, USP15 knockdown significantly reduced the protein levels of β-catenin, c-myc, and cyclin D1 in tumor tissue of nude mouse, consistent with the *in vitro* results

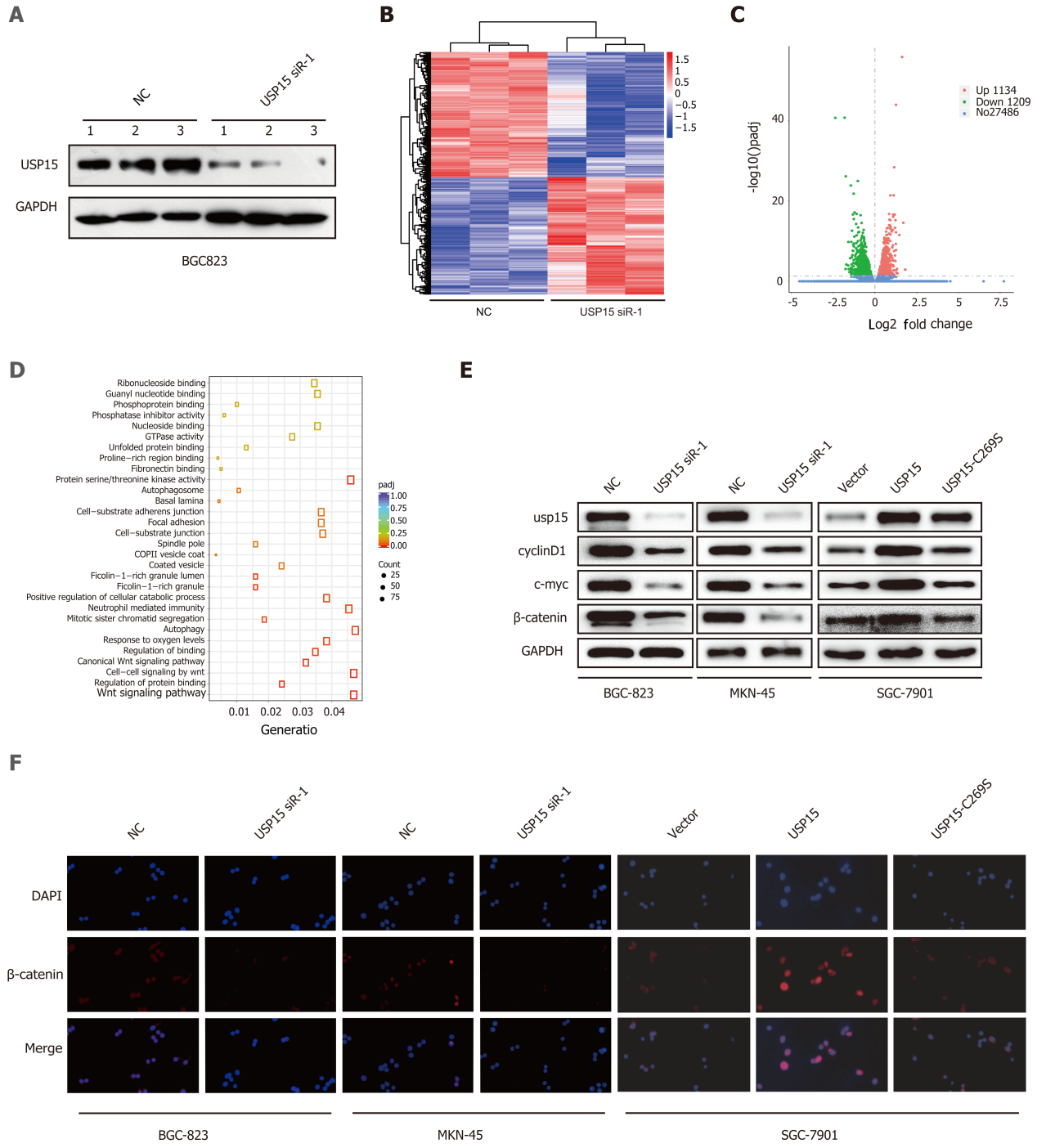


Figure 4 Ubiquitin-specific protease 15 regulates the Wnt/β-catenin pathway in gastric cancer. A: Western blotting confirmed the transfection efficiency of ubiquitin-specific protease 15 (USP15) knockdown in BGC-823 cell lines before RNA sequencing analysis; B: Heat map and hierarchical clustering based on the most differentially expressed genes (5739); C: Volcano plot illustrated differentially regulated gene expression. 1134 upregulated genes (red) and 1209 downregulated genes (green); D: Gene Ontology enrichment analyses of targets associated with USP15 for biological process, cellular component, and molecular function (top 10 most significantly affected categories are shown); E: Expression levels of USP15, β-catenin, c-Myc, and cyclin D1 in BGC-823 and MKN-45 cell lines transfected with USP15 knockdown or SGC-7901 cell lines transfected with USP15 overexpression; F: Immunofluorescence staining of β-catenin in BGC-823 and MKN-45 cell lines transfected with USP15 knockdown. Blue: DAPI; Red: β-catenin. Bar = 50 μm.

(Figure 6D).

DISCUSSION

In recent years, an increasing number of USP proteins have been reported to be critical to human cancers. For example, high expression of USP28 is related to the OS of patients with non-small cell lung cancer[27], while the expression of USP22 and USP11

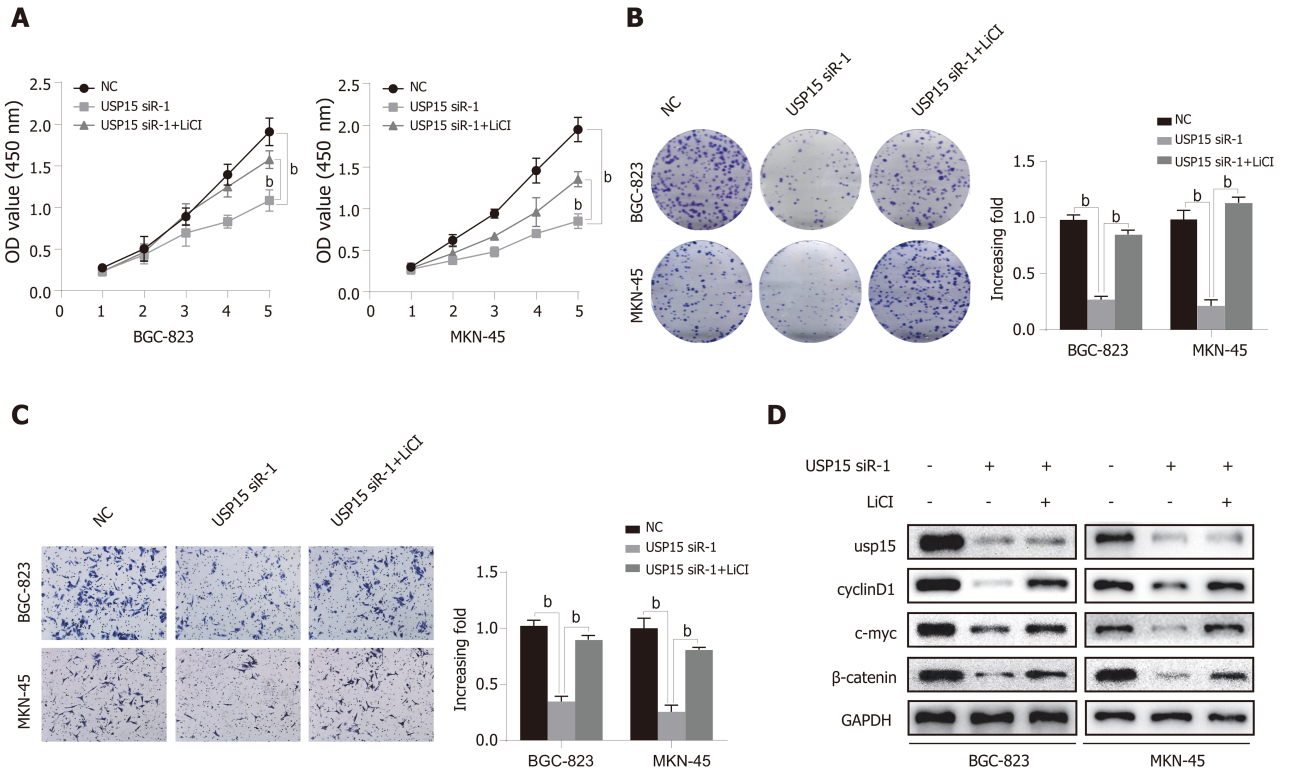


Figure 5 Lithium chloride partly reverses the effects of ubiquitin-specific protease 15 knockdown on gastric cancer progression. BGC-823 and MKN-45 cells transfected with ubiquitin-specific protease 15 (USP15) small interfering RNA-1 or negative control were incubated with or without lithium chloride (LiCl) (20 mmol/L). A, B: The Cell Counting Kit-8 (CCK-8) assay and colony formation assay evaluated cell proliferation ability; C: Transwell assays evaluated cell invasion ability; D: Western blotting detected the protein expression of β-catenin, c-Myc, and cyclin D1. $^{b}P < 0.01$, data are expressed as the mean ± standard error of the mean.

is related to the poor prognosis of breast cancer[28,29]. Two recent studies have shown that USP15 is upregulated in liver cancer and pancreatic ductal cell carcinoma[11,12]. In this study, IHC analyses showed that the high expression of USP15 was closely related to the depth of invasion, LNI, TNM stage, which indicated that USP15 acted as an oncogene, thereby promoting GC invasion, metastasis, and progression. In addition, the high expression of USP15 was related to the poor survival rate of GC patients, suggesting that USP15 is very important in the pathogenesis and development of GC, and could be used as a prognostic biomarker.

Similar to previous results[11,12], our study confirmed that USP15 was significantly associated with tumor cell proliferation *in vitro*. In addition, we also found that USP15 could participate in the tumor growth *in vivo*. Subsequently, we further found that USP15 can significantly promote the migration and invasion of GC cells *in vitro*. Migration and invasion, as the basic characteristics of malignant tumors, are the main reasons for the short survival time of cancer patients[30,31]. Mounting evidence has shown that tumor cells after EMT has high motility and aggressiveness, among which E-cadherin, N-cadherin, and vimentin are important molecular markers[32]. In addition, the epithelial marker E-cadherin is downregulated, while the mesenchymal markers vimentin and N-cadherin are upregulated during EMT[32]. As shown in our results, knockdown of USP15 resulted in upregulation of E-cadherin and downregulation of N-cadherin and vimentin, while overexpression of USP15 had the opposite effects, suggesting that USP15 can induce EMT in GC cells. The above findings indicated that USP15 may promote cell proliferation, migration, invasion and EMT process to become an oncogene of GC.

USP15 was related to a variety of cell signaling events, including transforming growth factor β (TGF-β)[13,33], constitutive photomorphogenesis 9 (COP9) signaling body[34], p53 signaling pathway[14], and nuclear factor kappa B (NF-kB)[35]. For example, USP15 promotes the stabilization of TGF-β receptor and its downstream signal transducers, thereby resulting in enhanced TGF-β signaling[13,36]. USP15 can protect the constituent subunits of cullin-RING ubiquitin ligase from self-ubiquitination and degradation *via* a stable cooperation with COP9-signalosome[34,37]. USP15 can stabilize MDM2 and negatively regulate the protein level of p53, and inactivation of USP15 can induce tumor apoptosis and improve the antitumor T-cell

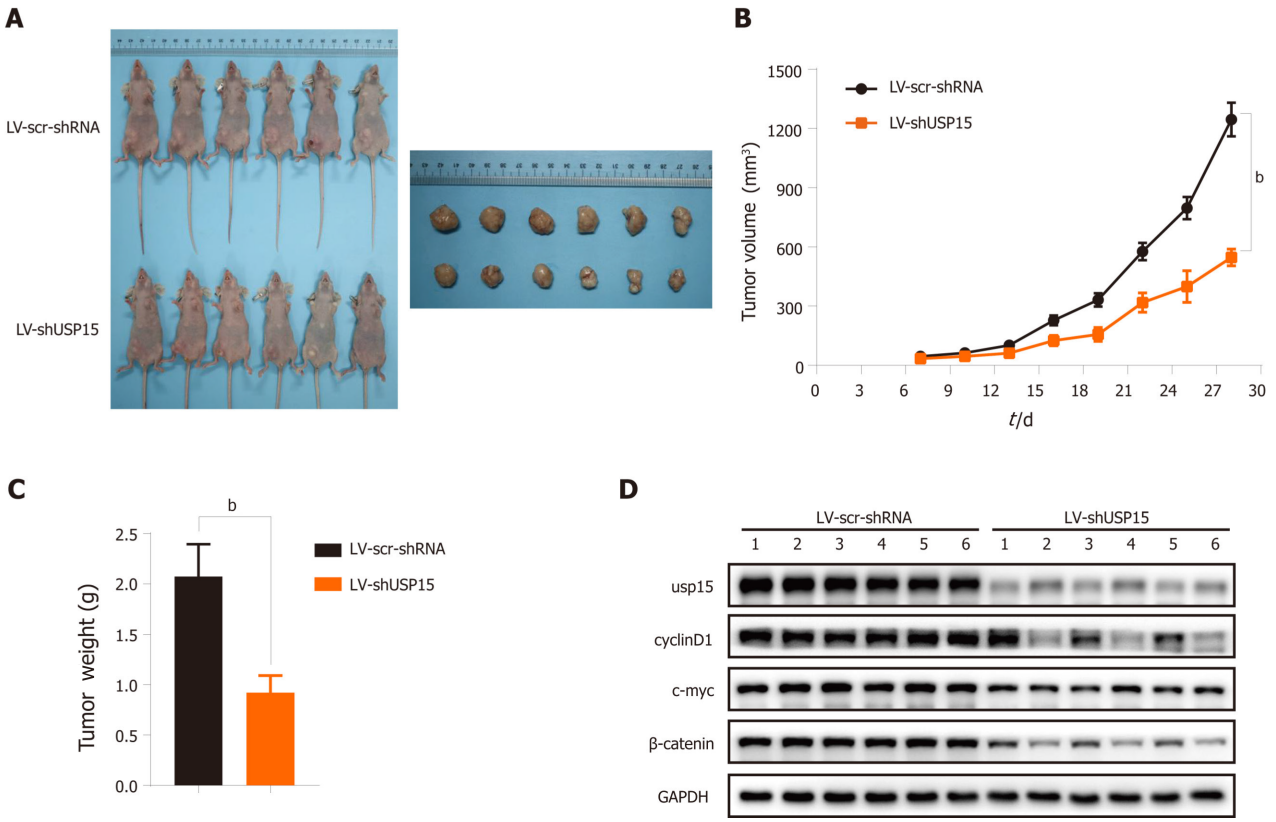


Figure 6 Ubiquitin-specific protease 15 silencing inhibits tumor growth *in vivo*. A: Cervical dislocation was used to sacrifice nude mice on day 29 and representative images of xenograft mouse samples were obtained (n = 6); B: Tumor volume was measured every 3 d and a growth curve was drawn; C: Tumor weight was measured when mice were sacrificed; D: Western blotting detected the protein expression of ubiquitin-specific protease 15, β-catenin, c-Myc, and cyclin D1 in tumor samples of two groups. $^{b}P < 0.01$, data are expressed as the mean ± standard error of the mean. USP15: Ubiquitin-specific protease 15.

response[14]. Another recent study showed that USP15 can effectively activate NF-κB by maintaining the stability of TAB2/3 differentially[38]. In our study, GO enrichment analysis based on RNA-Seq indicated that USP15 regulated the Wnt/β-catenin signaling pathway in GC. Previous studies have shown that abnormal activation of the Wnt/β-catenin pathway could promote the malignant progression of a variety of cancers, including GC[39,40]. Increased nuclear expression of β-catenin is an important sign of Wnt/β-catenin signaling pathway activation, which mainly depends on the transport of cytoplasmic β-catenin to the nucleus[39,40]. In our study, knockdown of USP15 significantly reduced the nuclear expression of β-catenin and downregulation of Wnt/β-catenin downstream genes in GC cells, while USP15 overexpression yielded opposite results, and there was no change in the USP15 C269S group (USP15 mutant), indicating that USP15 acted as a Wnt/β-catenin pathway activator. A rescue experiment by using LiCl (a Wnt/β-catenin pathway activator) showed that the effect of USP15 on GC progression was dependent on Wnt/β-catenin pathway. All of these findings suggest that USP15 contributes to GC progression by regulating the Wnt/β-catenin signaling pathway.

To the best of our knowledge, this study is the first to explore the clinical significance and molecular function of USP15 in GC. However, our research had some limitations. This was a retrospective study that included a small number of GC patients from a single center in our hospital, so there may have been a degree of bias. In the future, a large multicenter study should be conducted to verify our results. In addition, although GeneMANIA[41], a protein interaction bioinformatics website, predicts that USP15 can interact with some upstream proteins (CTNNB1, NUSAP1) of the Wnt/β-catenin pathway, the specific molecular mechanism problems need to be resolved in future research.

CONCLUSION

In conclusion, the results presented in our study demonstrated that USP15 was

upregulated in GC cells and tissues, and was associated with a poor prognosis in patients with GC. Furthermore, USP15 promoted cell proliferation, invasion, and EMT progression *via* the Wnt/β-catenin signaling pathway *in vitro* and promoted the growth of GC cells *in vivo*. All of our findings shed light on USP15 as a novel promising therapeutic target for understanding the pathogenesis of GC, providing new insights into the development of novel strategies for diagnosis and treatment from the bench to clinic.

ARTICLE HIGHLIGHTS

Research background

Ubiquitin-specific protease 15 (USP15) is an important member of the ubiquitin-specific protease (USP) family, whose expression is dysregulated in many types of cancer. However, the function role and the underlying mechanism of USP15 in gastric cancer (GC) progression have not yet been elucidated.

Research motivation

To explore the underlying mechanisms of GC development and discover biomarkers for the treatment of GC.

Research objectives

To investigate the role and potential mechanism of USP15 in GC.

Research methods

Bioinformatics databases and western blot analysis were utilized to determine the expression of USP15 in GC. Immunohistochemistry was performed to evaluate the correlation between expression of USP15 and clinicopathological characteristics of GC patients. A loss- and gain-of-function experiment was used to investigate the biological effects of USP15 on GC carcinogenesis. RNA sequencing analysis, immunofluorescence, and western blotting were performed to explore the potential mechanism by which USP15 exerted its oncogenic functions.

Research results

USP15 was upregulated in GC tissue and cell lines. The expression level of USP15 was positively correlated with clinical characteristics (tumor size, depth of invasion, lymph node involvement (LNI), tumor-node-metastasis (TNM) stage, perineural invasion, and vascular invasion), and was related to poor prognosis. USP15 knockdown significantly inhibited cell proliferation, invasion and epithelial-mesenchymal transition of GC *in vitro*, while overexpression of USP15 promoted these processes. Knockdown of USP15 inhibited tumor growth *in vivo*. Mechanistically, RNA-seq analysis showed that USP15 regulated the Wnt signaling pathway in GC. Western blotting confirmed that USP15 silencing led to significant downregulation of β-catenin and Wnt/β-catenin downstream genes (c-myc and cyclin D1), while overexpression of USP15 yielded the opposite results and USP15 mutation showed no change. Immunofluorescence indicated that USP15 promoted the nuclear translocation of β-catenin, suggesting activation of the Wnt/β-catenin signaling pathway, which may be the critical mechanism promoting GC progression. Finally, rescue experiments showed that the effects of USP15 on gastric cancer progression were dependent on the Wnt/β-catenin pathway.

Research conclusions

USP15 promotes cell proliferation, invasion, and EMT progression of GC *via* regulating the Wnt/β-catenin pathway.

Research perspectives

USP15 is expected to be a novel potential therapeutic target for GC.

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ORIGINAL ARTICLE

Retrospective Study

Feasibility of totally laparoscopic gastrectomy without prophylactic drains in gastric cancer patients

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Abstract

BACKGROUND

Prophylactic drains have been used to remove intraperitoneal collections and detect complications early in open surgery. In the last decades, minimally invasive gastric cancer surgery has been performed worldwide. However, reports on routine prophylactic abdominal drainage after totally laparoscopic distal gastrectomy are few.

AIM

To evaluate the feasibility performing totally laparoscopic distal gastrectomy without prophylactic drains in selected patients.

METHODS

Data of patients with distal gastric cancer who underwent totally laparoscopic distal gastrectomy with and without prophylactic drainage at China National Cancer Center/Cancer Hospital from February 2018 to August 2019 were reviewed. The outcomes between patients with and without prophylactic drainage were compared.

RESULTS

A total of 457 patients who underwent surgery for gastric cancer were identified. Of these, 125 patients who underwent totally laparoscopic distal gastrectomy were included. After propensity score matching, data of 42 pairs were extracted. The incidence of concurrent illness was higher in the drain group (42.9% *vs* 31.0%, $P = 0.258$). The overall postoperative complication rates were 19.5% and 10.6% in

the drain (n = 76) and no-drain groups (n = 49), respectively; there were no significant differences between the two groups (P > 0.05). The difference between the two groups based on the need for percutaneous catheter drainage was also not significant (9.8% *vs* 6.4%, P = 0.700). However, patients with a larger body mass index (≥ 29 kg/m^2) were prone to postoperative complications (P = 0.042). In addition, the number of days from surgery until the first flatus (4.33 ± 1.24 d *vs* 3.57 ± 1.85 d, P = 0.029) was greater in the drain group.

CONCLUSION

Omitting prophylactic drainage may reduce surgery time and result in faster recovery. Routine prophylactic drains are not necessary in selected patients. A prophylactic drain may be useful in high-risk patients.

Key Words: Gastric cancer; Prophylactic drainage; Totally laparoscopic gastrectomy; Enhanced recovery after surgery; Minimally invasive surgery; Early gastric cancer

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Core Tip: We reviewed the outcomes of 125 consecutive patients with distal gastric cancer who underwent totally laparoscopic distal gastrectomy with and without prophylactic drainage at China National Cancer Center/Cancer Hospital from February 2018 to August 2019. We found that performing totally laparoscopic gastrectomy without prophylactic drains in selected patients is possible. It significantly improved postoperative comfort and did not increase the risk of postoperative complications.

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INTRODUCTION

In the last decade, gastric cancer has been one of the most frequently occurring malignancies worldwide, with about one million new cases of gastric cancer in 2017. It is the fifth most common malignancy and the third highest malignant tumor, with an estimated 783000 deaths[1]. In China, there were approximately 677000 new gastric cancer cases in 2015. This accounted for half of the new gastric cancer cases worldwide [2].

In 1994, Kitano *et al*[3] reported the first case of laparoscopic assisted distal gastrectomy (LADG) with D2 lymphadenectomy[3]. A recent multi-center clinical study in South Korea also confirmed that the operation was safe and effective[4]. With the development of surgical instruments and technology, early minimally invasive gastric cancer surgery has been widely performed worldwide. Meanwhile, the interim results of a class 01 clinical trial led by China's Southern Hospital showed that the efficacy of laparoscopic surgery for advanced distal gastric cancer was comparable to that of open surgery[5].

The development of laparoscopic gastric cancer surgery has led to its emergence as a treatment modality for distal gastric cancer. Compared with laparoscopic assisted surgery, totally laparoscopic distal gastrectomy (TLDG) is an intra-cavitary anastomosis, which does not require an auxiliary small incision. The reconstruction of TLDG anastomosis is safer, regardless of tumor location, with a lower incidence of incision problems than LADG. Moreover, it can be performed more effectively in obese patients[6,7].

Prophylactic drains have been used to remove intraperitoneal collections and detect complications early. However, numerous trials have failed to demonstrate a reduction in postoperative complications by routine drainage in gastrointestinal surgery[8]. Several studies performed after open gastrectomy or LADG concluded that the prophylactic use of drains did not significantly improve postoperative outcomes.

because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest to disclose.

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However, there are few studies on routine prophylactic drainage after TLDG.

In the current retrospective study, we compared the outcomes of patients who underwent TLDG with and without drainage to clarify the value of routine prophylactic drainage in uncomplicated TLDG procedures for distal gastric cancer.

MATERIALS AND METHODS

Patients

We reviewed the outcomes of 457 consecutive patients with distal gastric cancer who underwent TLDG with and without prophylactic drainage at China National Cancer Center/Cancer Hospital from February 2018 to August 2019. Among them, 145 patients who underwent proximal gastrectomy or total gastrectomy, 159 patients who underwent laparoscopic assisted surgery, 23 who underwent open gastrectomy (including four cases converted from laparoscopic surgery), and five who underwent simultaneous surgery for other diseases such as choledocholithiasis (n = 1), ovarian tumor (n = 1), and pancreatic tail (n = 3) were excluded. Finally, a total of 125 patients were included in this study. They were assigned to a drain or no-drain group according to their operation records. The drain group comprised 76 patients who underwent TLDG with routine prophylactic drainage, and the no-drain group comprised 49 patients who underwent TLDG without routine prophylactic drainage (Figure 1).

Totally laparoscopic distal gastrectomy

The extent of gastrectomy and lymph node dissection were determined based on the Japanese gastric cancer treatment guidelines[9]. The surgeon was on the left side of the patient to finish laparoscopic ligation and division, and the first assistant was positioned on the opposite side. A cameraman stood between the patient' s legs. A five-port system (*i.e.*, two 5 mm and three 12 mm ports) was used for each totally laparoscopic distal gastrectomy. Ten-millimeter flexible laparoscopes were used, with CO_2 pressure maintained at 13-15 mmHg.

The operator was on the left side of the patient to perform Billroth-I reconstruction using a modified delta-shaped anastomosis[10] or overlap anastomosis[8]. Billroth-II or Roux-en-Y reconstruction was performed on the right side of the patients.

Postoperative management

Patients in both groups were administered prophylactic antibiotics 30 min before surgery. The decision of whether to use a prophylactic drain was made by the surgeon. Oral intake of water was initiated on the first day after surgery. A soft diet was initiated after the patient could tolerate liquid meals, and postoperative upper gastrointestinal contrast confirmed the absence of anastomotic leakage.

Outcome assessment

The clinical, operative, and pathological variables were compared between the two groups based on the information obtained from our prospectively collected surgical database. Early postoperative complications (occurring on postoperative days 0-30) were graded using the Clavien–Dindo classification. Early postoperative complications requiring medical, radiological, or surgical interventions (grade 2 or higher) were regarded as events. The risk for the occurrence of postoperative complications was also assessed.

Statistical analyses

All values are expressed as the mean ± SD. The χ^2 test and Student's t test were used to compare the categorical and continuous variables, respectively. For categorical data, the chi-squared test or Fisher's exact test was performed. A P value of < 0.05 was considered significant. Statistical analyses were performed using Statistic Package for Social Science. 20.

Propensity score matching

Multiple factor logistic regression models were used to calculate the propensity score for each patient to balance the following covariates: Age, sex, body mass index (BMI), abdominal operation history, smoking history, drinking history, concurrent illness, American Society of Anesthesiologists classification, operation time, estimated blood loss, primary tumor stage, regional lymph node stage, tumor size, and number of

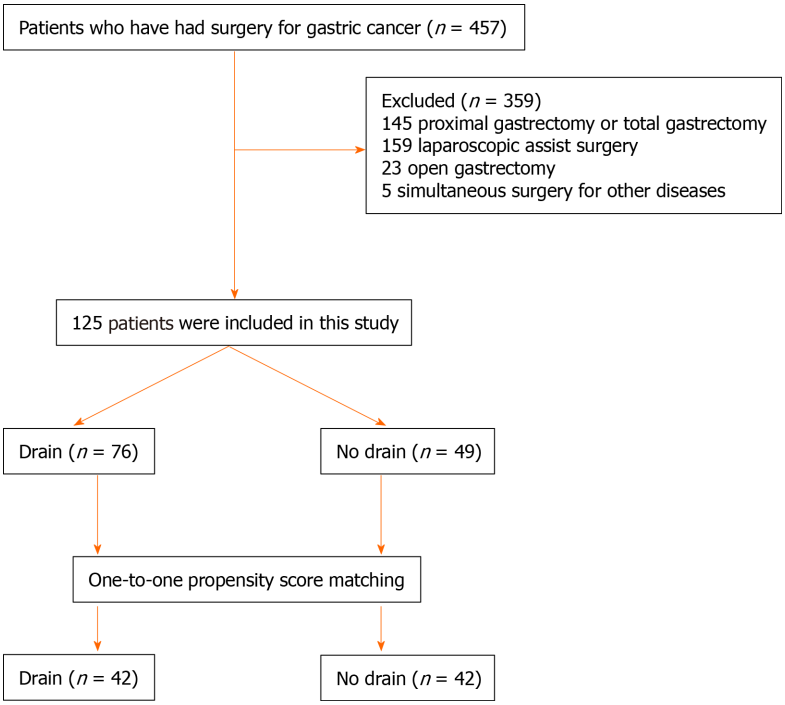


Figure 1 Flow chart of the patients assessed in this study.

retrieved lymph nodes. We imposed a caliper width of 0.1 of the standard deviation of the logistic propensity score.

RESULTS

Patient characteristics

Table 1 shows the clinical characteristics of patients undergoing TLDG with or without a prophylactic drain. No significant differences were observed in patient sex, age, BMI, American Society of Anesthesiologists classification, abdominal operation history, smoking history, drinking history, concurrent illness primary tumor stage, or regional lymph node stage between the two groups after propensity score matching (PSM).

Operative findings

The operative outcomes are summarized in Table 2. The drain group had a longer operating time than the no-drain group (198.4 ± 41.0 min *vs* 164.0 ± 37.0 min, $P < 0.001$). Mean estimated blood loss and intraoperative blood transfusion were similar between the two groups. There were no significant differences between the two groups in terms of the number of retrieved lymph nodes and tumor size ($P > 0.05$). After PSM, no significant differences were noted in operating time between the drain and no-drain groups.

Recovery

The recovery outcomes are listed in Table 3. The number of days from surgery to the initiation of soft diet (5.34 ± 2.27 d *vs* 4.17 ± 2.13 d, $P = 0.036$) and to first flatus (4.29 ± 1.45 d *vs* 3.55 ± 1.83 d, $P = 0.041$) were greater in the drain group. There were no significant differences in the time to ambulation or length of postoperative hospital stay (8.15 ± 2.9 d *vs* 6.77 ± 2.3 d, $P = 0.219$) between the two groups. Postoperative C-reactive protein levels (8.24 ± 4.47 mg/L *vs* 8.67 ± 5.97 mg/L, $P > 0.05$) and postoperative maximum body temperature (Tmax) (37.6 ± 0.6 ℃ *vs* 37.5 ± 0.4 ℃, $P > 0.05$) were similar between the two groups. After PSM, only the number of days from surgery to first flatus (4.33 ± 1.24 d *vs* 3.57 ± 1.85 d, $P = 0.029$) was greater in the drain group.

Short-time outcomes

Postoperative patient complications are listed in Table 4. No mortality was recorded in

Table 1 Characteristics of patients who underwent totally laparoscopic distal gastrectomy with or without prophylactic drain

	ALL patients			Propensity-matched patients		
Characteristic	Drain (*n* = 76)	No drain (*n* = 49)	*P* value	Drain (*n* = 42)	No drain (*n* = 42)	*P* value
Sex (M/F)	54/22	33/16	0.660	31/11	29/13	0.629
Age	57.58 ± 9.90	54.14 ± 12.63	0.092	57.4 ± 9.9	58.1 ± 10.8	0.739
BMI (kg/m^2)	24.71 ± 3.76	24.64 ± 3.72	0.915	24.3 ± 3.5	24.4 ± 2.7	0.879
ASA (1/2/3), *n* (%)			0.562			0.565
1	1 (1.3)	0 (0.0)		1 (2.4)	0 (0.0)	
2	70 (92.1)	44 (89.8)		38 (90.5)	38 (90.5)	
3	5 (6.6)	5 (10.2)		3 (7.1)	4 (9.5)	
pT stage, *n* (%)			0.605			0.805
T1	39 (52.0)	24 (49.0)		20 (47.6)	20 (47.6)	
T2	10 (13.3)	11 (22.4)		6 (14.3)	10 (23.8)	
T3	9 (12)	5 (10.2)		6 (14.3)	4 (9.5)	
T4a	17 (22.7)	9 (18.4)		10 (23.8)	8 (19)	
pN stage, *n* (%)			0.888			0.760
N0	34 (44.7)	20 (40.8)		16 (38.1)	18 (42.9)	
N1	16 (21.1)	12 (24.5)		9 (21.4)	11 (26.2)	
N2	14 (18.4)	7 (14.3)		9 (21.4)	5 (11.9)	
N3	12 (15.8)	10 (20.4)		8 (19)	8 (19)	
Previous abdominal operation, *n* (%)	13 (17.1)	13 (26.5)	0.205	6 (14.3)	10 (23.8)	0.266
Neoadjuvant chemotherapy, *n* (%)	5 (6.6)	2 (4.1)	0.704	4 (9.5)	2 (4.8)	0.676
Concurrent illness, *n* (%)	34 (44.7)	14 (28.6)	0.070	18 (42.9)	13 (31.0)	0.258

ASA: American Society of Anesthesiologists; pT: Primary tumor; pN: Regional lymph node; BMI: Body mass index.

Table 2 Operative findings

Variable	All patients			Propensity-matched patients		
	Drain (*n* = 76)	No drain (*n* = 49)	*P* value	Drain (*n* = 42)	No drain (*n* = 42)	*P* value
Operation time (min)	198.4 ± 41.0	164.0 ± 37.0	< 0.001	180.2 ± 33.4	168.0 ± 36.7	0.113
Estimated blood loss (mL)	85.3 ± 80.7	70.82 ± 51.5	0.267	72.9 ± 45.8	73.8 ± 54.4	0.931
Intraoperative blood transfusion, *n* (%)	2 (2.6)	2 (4.1)	0.645	1 (2.4)	2 (4.8)	1.000
Tumor size (cm)	3.5 ± 1.6	3.6 ± 1.5	0.664	3.6 ± 1.7	3.5 ± 1.4	0.839
No. of retrieved lymph nodes	36.7 ± 13.7	39.1 ± 14.2	0.346	40.0 ± 11.2	40.0 ± 15.1	0.923

either group. The overall postoperative complication rates were 15.8% and 10.2% in the drain and no-drain groups, respectively ($P > 0.05$). No anastomotic bleeding, anastomotic leakage, lymph leakage, ileus, or pancreatic fistula occurred in either group. Clavien-Dindo grade 3 complications included duodenal stump leakage ($n = 2$), anastomotic leakage ($n = 2$), intra-abdominal abscess ($n = 2$), and intra-abdominal bleeding ($n = 1$) in the drainage group. The need for percutaneous catheter drainage (PCD) was not significantly different between the groups (9.8% *vs* 6.4%, $P = 0.700$). After PSM, no significant differences were noted in the complications between the drain and no-drain groups.

Risk assessment for the occurrence of postoperative complication

Postoperative complication risk factors are listed in Table 5. Between the two groups,

Table 3 Recovery

Variable	All patients			Propensity-matched patients		
	Drain (*n* = 76)	No drain (*n* = 49)	*P* value	Drain (*n* = 42)	No drain (*n* = 42)	*P* value
Time to ambulation, POD	2.51 ± 1.34	2.98 ± 1.39	0.064	2.90 ± 1.54	3.07 ± 1.44	0.610
Time to first flatus, POD	3.97 ± 1.24	3.55 ± 1.79	0.122	4.33 ± 1.24	3.57 ± 1.85	0.029
Time to first eating of soft diet, POD	4.70 ± 2.17	4.14 ± 2.09	0.159	5.02 ± 1.88	4.17 ± 2.20	0.058
Postoperative hospital stay	7.88 ± 3.96	6.73 ± 5.13	0.164	7.93 ± 4.98	6.81 ± 5.50	0.331
CRP	7.54 ± 4.38	8.53 ± 5.91	0.286	7.66 ± 3.89	8.71 ± 5.95	0.339
Tmax	37.6 ± 0.5	37.5 ± 0.4	0.239	37.60 ± 0.60	37.48 ± 0.40	0.300

POD: Postoperative days.

Table 4 Postoperative complications

Complication, *n*	All patients			Propensity-matched patients		
	Drain (*n* = 76), *n* (%)	No drain (*n* = 49), *n* (%)	*P* value	Drain (*n* = 42), *n* (%)	No drain (*n* = 42), *n* (%)	*P* value
Total	12(15.8)	5 (10.2)	0.374	8 (19.0)	4 (9.5)	0.212
Clavien–Dindo grade II	4 (5.2)	2 (4.0)		3 (7.2)	1 (2.4)	
Incision	1 (1.3)	1 (2.0)		0 (0.0)	0 (0.0)	
System complications	1 (1.3)	1 (2.0)		1 (2.4)	1 (2.4)	
Abdominal effusion	2 (2.6)	0 (0.0)		2 (4.8)	0 (0.0)	
Clavien–Dindo grade III	8 (10.6)	3 (6.0)		5 (12)	3 (7.2)	
Duodenal stump leakage	2 (2.6)	0 (0.0)		2 (2.4)	0 (0.0)	
Anastomotic Leakage	2 (2.6)	0 (0.0)		0 (0.0)	0 (0.0)	
Intra-abdominal bleeding	1 (1.3)	0 (0.0)		1 (2.4)	0 (0.0)	
Intra-abdominal abscess	3 (3.9)	2 (4.0)		2 (2.4)	2 (2.4)	
Pleural effusion	0 (0.0)	1 (2.0)		0 (0.0)	1 (2.4)	
Mortality	0 (0.0)	0 (0.0)	1	0 (0.0)	0 (0.0)	1

PCD: Percutaneous catheter drainage.

no significant differences were observed in most variables. However, the patients with a larger BMI had a higher possibility of postoperative complications (27.44 ± 3.92 *vs* 24.25 ± 3.53, $P = 0.01$). In addition, we identified that patients with a BMI ≥ 29 kg/m^2 were prone to postoperative complications ($P = 0.042$). A prophylactic drain may be useful in patients with a higher risk, larger BMI, or more concurrent illness. Prophylactic drains was not an independent risk factor for postoperative complications.

DISCUSSION

Since 2015, totally laparoscopic surgery has been widely used in clinical practice, although there are few reports on whether totally laparoscopic surgery requires prophylactic drains[10,11]. Most studies on prophylactic drains were based on open gastrectomy. Cochrane review included four single-institution, randomized controlled trials that sought to evaluate the role of prophylactic drain placement in gastric resection for gastric cancer[12-14]. In this study, we reviewed the clinicopathological data of patients with gastric cancer during the past 2 years and found that routine prophylactic drains were not necessary in selected patients. To minimize the risk of confounding variables, PSM was used. Routine prophylactic drains are not necessary in all patients. A prophylactic drain may be useful in patients at higher risk.

Table 5 Risk assessment for the occurrence of postoperative complication

Variable	Postoperative complications (+) (*n* = 17)	Postoperative complications (-) (*n* = 108)	*P* value
Sex			0.584
Male	13	74	
Female	4	34	
Age	59.59 ± 9.62	55.70 ± 11.30	0.182
BMI (kg/m^2), *n* (%)	27.44 ± 3.92	24.25 ± 3.53	0.001
≥ 29	5 (38.5)	10 (13.3)	0.042
< 29	8 (61.5)	65 (86.7)	
ASA (1/2/3), *n* (%)			0.769
1	0 (0.0)	1 (100.0)	
2	15 (13.2)	99 (86.8)	
3	2 (20.0)	8 (80.0)	
Preoperative ALB (g)	39.62 ± 4.65	40.18 ± 5.86	0.709
Preoperative HGB (g/L)	136.59 ± 17.77	135.26 ± 19.36	0.791
pT stage, *n* (%)			0.776
T1	7 (38.5)	56 (49.3)	
T2	4 (23.1)	17 (20.0)	
T3	3 (15.4)	11 (8.0)	
T4a	3 (23.1)	23 (22.7)	
pN stage, *n* (%)			0.872
N0	8 (38.5)	46 (45.3)	
N1	3 (15.4)	25 (22.7)	
N2	3 (23.1)	18 (13.3)	
N3	3 (23.1)	19 (18.7)	
Previous abdominal operation			0.103
Yes	1	25	
No	16	83	
Neoadjuvant chemotherapy			0.234
Yes	2	5	
No	15	103	
Concurrent illness			0.800
Yes	7	41	
No	10	67	
Drain, *n* (%)	12 (15.8)	64 (84.2)	0.374
No drain, *n* (%)	5 (10.2)	44 (89.8)	
Type of reconstruction, *n* (%)			0.357
Billroth I	4 (30.8)	32 (36.0)	
Billroth II	10 (61.5)	69 (64.0)	
Roux-en-Y	3 (7.7)	7 (0.0)	
Operative time (min)	195.82 ± 49.12	183.16 ± 41.69	0.258
Blood loss (mL)	62.94 ± 42.54	82.22 ± 74.07	0.298

ASA: American Society of Anesthesiologists; BMI: Body mass index; ALB: Albumin; HGB: Hemoglobin; pT: Primar tumor; pN: Regional lymph node.

Prophylactic drains have been used to enhance early detection of complications, prevent collection of fluid, reduce morbidity and mortality, and decrease the duration of hospital stay[15,16]. The present study results showed that there was no significant difference between the two groups in terms of postoperative hospital stay. The length of the postoperative hospital stay in the no-drain group was shorter than that in the drain group (7.93 ± 4.98 d *vs* 6.81 ± 5.50 d, $P > 0.05$). Among the 17 patients who experienced postoperative complications, there was also no significant difference between the two groups in terms of postoperative hospital stay. This result was different from that of Hirahara *et al*[10] study. In addition, omitting prophylactic drainage significantly improved the postoperative comfort of patients due to an earlier flatus (4.33 ± 1.24 d *vs* 3.57 ± 1.85 d, $P < 0.05$).

Moreover, the application of prophylactic drains did not reduce the incidence of complications, and the rate of complications was even higher in the drain group. However, there was no statistically significant difference between the two groups (19.0% *vs* 9.5%, $P > 0.05$). Through risk assessment, we identified that patients with a BMI ≥ 29 kg/m^2 are prone to postoperative complications ($P = 0.042$). More visceral fat may make surgery more difficult. Thus, prophylactic drain is recommended for patients with a BMI > 29 kg/m^2.

For patients with mild symptoms, administration of broad-spectrum antibiotics may be a good conservative management strategy. However, patients with severe symptoms need PCD. In the current study, postoperative complications were recognized in approximately 15% of patients. Two cases of duodenal stump leakage and two cases of intra-abdominal abscess occurred in the drain group, all of which required PCD. In the no-drain group, two cases of intra-abdominal abscess and one case of pleural effusion needed PCD. There was no significant difference between the two groups. Prophylactic drains do not alter the rates of secondary drainage procedures. Thus, omitting prophylactic drains during gastric cancer surgery did not increase the risk of PCD postoperatively. Similarly, in a study by Lee *et al*[16], omitting prophylactic drains did not increase the risk of PCD postoperatively, while male sex, older age, and longer operative time were identified as independent risk factors for postoperative PCD in patients without prophylactic drains.

CONCLUSION

In conclusion, omitting the use of prophylactic drains in selected patients during surgery for gastric cancer is feasible. It can significantly improve the postoperative comfort of patients and does not increase the risk of postoperative complications.

ARTICLE HIGHLIGHTS

Research background

Prophylactic drains have been used to remove intraperitoneal collections and detect complications early in open surgery. In the last decades, minimally invasive gastric cancer surgery has been performed worldwide. However, reports on routine prophylactic abdominal drainage after totally laparoscopic distal gastrectomy are few.

Research motivation

To evaluate the feasibility of performing totally laparoscopic distal gastrectomy without prophylactic drains in selected patients.

Research objectives

To evaluate the feasibility of performing totally laparoscopic distal gastrectomy without prophylactic drains in selected patients.

Research methods

Data of patients with distal gastric cancer who underwent totally laparoscopic distal gastrectomy with and without prophylactic drainage at China National Cancer

Center/Cancer Hospital from February 2018 to August 2019 were reviewed.

Research results

After PSM, data of 42 pairs were extracted. The incidence of concurrent illness was higher in the drain group (42.9% *vs* 31.0%, P = 0.258). The overall postoperative complication rates were 19.5% and 10.6% in the drain (n = 76) and no-drain groups (n = 49), respectively; there were no significant differences between the two groups (P > 0.05). The difference between the two groups based on the need for percutaneous catheter drainage was also not significant (9.8% *vs* 6.4%, P = 0.700). However, patients with a larger body mass index (≥ 29 kg/m^2) were prone to postoperative complications (P = 0.042). In addition, the number of days from surgery until the first flatus (4.33 ± 1.24 d *vs* 3.57 ± 1.85 d, P = 0.029) was greater in the drain group.

Research conclusions

Omitting prophylactic drainage may reduce surgery time and result in faster recovery. Routine prophylactic drains are not necessary in selected patients. A prophylactic drain may be useful in high-risk patients.

Research perspectives

Omitting the use of prophylactic drains can significantly improve the postoperative comfort of patients and does not increase the risk of postoperative complications.

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CORRECTION

Correction to "Downregulation of FoxM1 inhibits the viability and invasion of gallbladder carcinoma cells, partially dependent on the induction of cellular senescence"

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Author contributions: Tao J and Xu XS revised the manuscript; Song YZ provided the raw data; Liu C designed the study.

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Abstract

We corrected the mistake of Figure 3, and replaced the incorrect images with the correct ones. The "adenovirus" was a typographical error in writing, and should be revised to "lentivirus".

Key Words: Correction; Unintentional; Mistake; Error; Sorting; Figure

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Core Tip: We corrected the mistake of Figure 3 and manuscript text.

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TO THE EDITOR

We found a mistake in Figure 3[1]. This is an unintentional error that occurred when sorting through the images. We have replaced the incorrect images with the correct

Peer-review report's scientific quality classification

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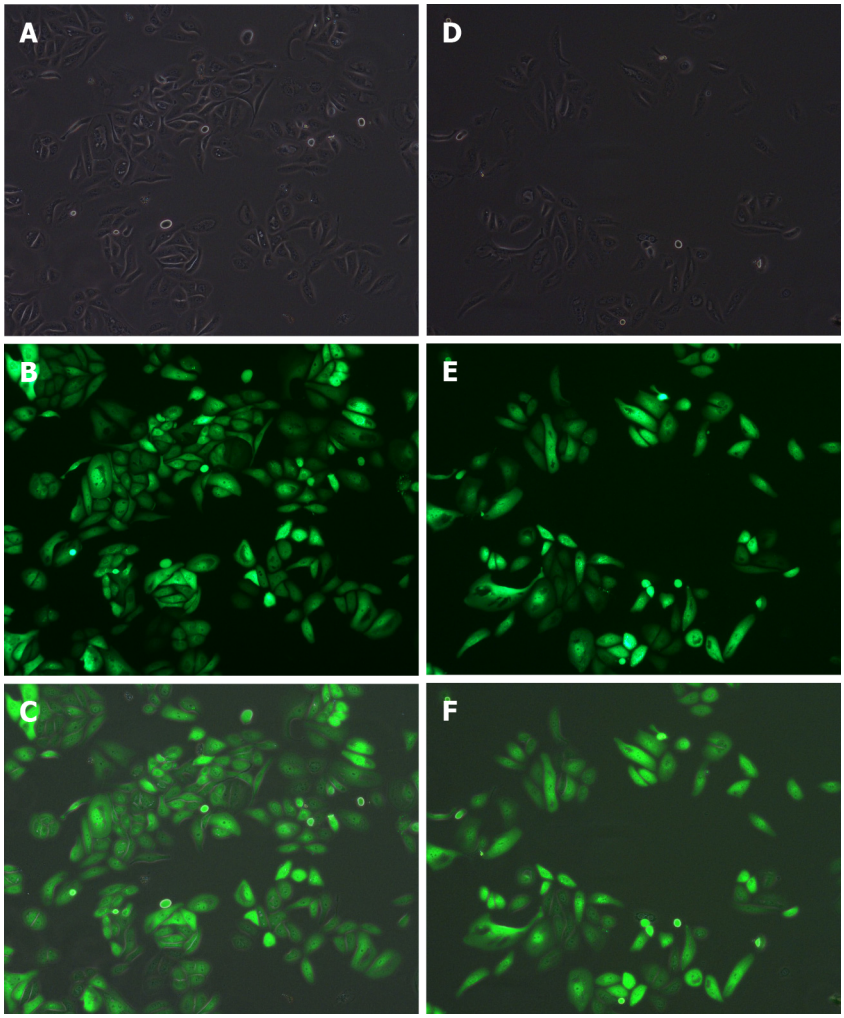


Figure 1 Representative photograph (100 ×) showing recombinant lentivirus transfection efficiency evaluated by fluorescence microscopy (transfected with the negative control, top; transfected with the shF1822, bottom). A and D: Light microscopy; B and E: Fluorescence microscopy; C and F: Superimposed image of the two images.

Figure 1. This technical error does not change the meaning of the picture or the conclusion of the manuscript. On the other hand, we only used lentivirus in the experiment. The "adenovirus" was a typographical error in writing. We mostly used "lentivirus" in the manuscript. "Adenovirus" should be revised to "lentivirus" in P9497 right column, line 11 and line 27; P9498 right column, line 26; P9499 right column, line 47; Figure 3 and Figure 4 captions. We apologize for our unintentional mistakes, which caused great inconvenience.

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LETTER TO THE EDITOR

Impact of COVID-19 on the clinical status of patients with Wilson disease

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Abstract

The coronavirus disease 2019 (COVID-19) pandemic has greatly impacted health systems. Many guidelines on chronic liver diseases have been released to optimize the use of medical resources and patient management. However, most of these guidelines have been established through expert consensus because the existing data do not provide strong evidence for developing effective recommendations. As Wilson disease (WD) is a rare chronic liver disease, the impact of COVID-19 on the clinical status of patients with WD is unclear. The present study showed a marked shortage of medical resources for clinically managing patients with WD during the pandemic. Although patients with WD who consistently took anticopper therapy showed no significant differences in hepatic and extrahepatic markers before and after the pandemic, their complication incidences, especially the infection incidence, were significantly increased during the study period. Therefore, patients with WD should be encouraged to adhere to anticopper therapy and be closely monitored to prevent infections and other complications. The present study provides a clinical basis for further managing WD during the pandemic.

Key Words: Coronavirus disease 2019; Wilson disease; Clinical status; Complications; Infections; Anticopper therapy

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Core Tip: The coronavirus disease 2019 (COVID-19) pandemic has had a long-lasting impact on the quality of care for patients with cirrhosis. Although many guidelines have been released for the rational use of medical resources, few clinical data are available to support these guidelines. The clinical features of patients with Wilson disease during the COVID-19 pandemic remain unclear. We compared the clinical features of patients with Wilson disease before and after the pandemic to clarify the impact of COVID-19 on these patients and provide a basis for their clinical management.

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TO THE EDITOR

Many countries have enforced social distancing and strict stay-at-home strategies to reduce the spread of coronavirus disease 2019 (COVID-19). However, these measures often negatively affect patients with other diseases[1,2]. Many guidelines on chronic liver diseases have been released to optimize the use of medical resources and patient management[3]. Most of these guidelines have been established through expert consensus because the existing data do not provide strong evidence for developing effective recommendations.

Given the high copper deposition in the livers of patients with Wilson disease (WD), these patients often develop liver injury and cirrhosis. Because WD has clinical features that are distinct from those of liver diseases caused by other etiologies and medical resources have been in short supply during the pandemic, the clinical features of patients with WD should be examined to improve their management. Therefore, we conducted a before–after study to investigate the clinical features of these patients before and during COVID-19.

We reviewed the medical records of patients with WD who were hospitalized for routine office visits or emergency visits at the First Affiliated Hospital of Guangdong Pharmaceutical University from 1 January 2018 to 3 September 2020. In China, the diagnostic criteria for WD are similar to those of the diagnostic scoring system for WD. During the COVID-19 pandemic, the number of WD inpatient visits dropped from 198 to 95, indicating a 52.02% decrease from the number of WD inpatient visits during the same period in 2019. These data indicate that the ongoing pandemic has led to a marked shortage of medical resources for clinically managing patients with WD. Medical data on 68 patients with WD who were hospitalized at our hospital during and before the pandemic were analyzed. All of these patients underwent anticopper therapy during the pandemic. Most of them (83.82%) had developed cirrhosis before the pandemic, and none had COVID-19.

The hepatic and extrahepatic status of patients who consistently used anticopper therapy during the pandemic did not significantly deteriorate (Table 1). However, owing to lifestyle changes and delayed screening for complications during the pandemic, the complication incidence increased significantly in these patients during the study period (23.53% *vs* 11.76%, $P = 0.021$). Notably, most complications (22/24) occurred in patients with WD-associated cirrhosis. Among the complications, infections were the most prevalent (11.8% *vs* 1.5%, $P = 0.016$). Although the community mitigation measures for COVID-19 are thought to reduce the incidence of respiratory infections in the general population[4], our data showed that the incidence of respiratory infections in patients with WD increased during the pandemic (7.4% *vs* 0%, $P = 0.063$).

Following the COVID-19 outbreak, the Chinese government implemented strong strict measures, and most citizens, except those involved in essential services, were ordered to stay at home. These measures helped keep the pandemic under control in China. However, the lockdown and movement restrictions often led to reduced physical activity, prolonged sedentary behaviors, imbalanced nutritional intake, poor mental health and delayed routine follow-up visits in these patients[5]. These changes were associated with cirrhosis-associated immune dysfunction and accounted for the

Table 1 Clinical features and complications in patients with Wilson disease before and after coronavirus disease 2019

	Before COVID-19 (*n* = 68)	After COVID-19 (*n* = 68)	*P* value
Demographic characteristics			
Age (yr)	28.00 (23.00–33.00)		-
Male sex	37 (54.41)		-
Hepatic features			
Elevated ALT (> 40 U/L)	16 (23.53)	12 (17.65)	0.424
Elevated AST (> 35 U/L)	13 (19.12)	17 (25.00)	0.388
Elevated bilirubin (> 17.1 μmol/L)	15 (22.06)	13 (19.12)	0.754
Hypoproteinemia (albumin < 35 g/L)	10 (14.71)	12 (17.65)	0.774
Elevated PT (> 15 s)	11 (16.18)	13 (19.12)	0.791
Elevated INR (> 1.5)	1 (1.47)	2 (2.94)	1.000
Child-Pugh			1.000
A	64 (94.12)	65 (95.59)	
B/C	4 (5.88)	3 (4.41)	
Cirrhosis	57 (83.82)	57 (83.82)	1.000
Extrahepatic features			
Neurological manifestations	50 (73.5)	49 (72.1)	1.000
Psychiatric manifestations	3 (4.4)	4 (5.9)	1.000
Kayser-Fleischer ring	32 (47.1)	35 (51.5)	0.375
Splenomegaly/splenectomy	45 (66.2)	47 (69.1)	0.688
Complications			
Any complication	8 (11.76)	16 (23.53)	0.021
Ascites	2 (1.5)	5 (7.4)	0.375
Infections	1 (1.5)	8 (11.8)	0.016
Respiratory infection	0 (0)	5 (7.4)	0.063
Urinary infection	0 (0)	1 (1.5)	1.000
Gastrointestinal infection	1 (1.5)	2 (2.9)	1.000
SBP	0 (0)	1 (1.5)	1.000
PVT	0 (0)	0 (0)	-
Gastroesophageal varices	5 (7.4)	7 (10.3)	0.500
Variceal bleeding	0 (0)	1 (1.5)	1.000
Hepatic encephalopathy	0 (0)	0 (0)	-
Renal impairment	0 (0)	0 (0)	-
Liver failure	0 (0)	0 (0)	-
HCC	0 (0)	0 (0)	-

Data are presented as medians (interquartile ranges) or *n* (%). ALT: Alanine transaminase; AST: Aspartate transaminase; HCC: Hepatocellular carcinoma; INR: International normalized ratio; PT: Prothrombin time; PVT: Portal vein thrombosis; SBP: Spontaneous bacterial peritonitis; COVID-19: Coronavirus disease 2019.

high infection risk[6].

In conclusion, the hepatic and extrahepatic status of patients with WD who adhered strictly to their anticopper therapy during the COVID-19 pandemic did not significantly worsen, but the complication incidence — especially the infection incidence — increased significantly. Therefore, patients with WD should be encouraged to adhere

to anticopper therapy and be closely monitored to prevent infections and other complications.

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REVIEW

Update on the association of hepatitis B with intrahepatic cholangiocarcinoma: Is there new evidence?

Nikolaos Fragkou, Lazaros Sideras, Panteleimon Panas, Christos Emmanouilides, Emmanouil Sinakos

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Abstract

Intrahepatic cholangiocarcinoma (iCCA) is a subgroup of cholangiocarcinoma that accounts for about 10%-20% of the total cases. Infection with hepatitis B virus (HBV) is one of the most important predisposing factors leading to the formation of iCCA. It has been recently estimated based on abundant epidemiological data that the association between HBV infection and iCCA is strong with an odds ratio of about 4.5. The HBV-associated mechanisms that lead to iCCA are under intense investigation. The diagnosis of iCCA in the context of chronic liver disease is challenging and often requires histological confirmation to distinguish from hepatocellular carcinoma. It is currently unclear whether antiviral treatment for HBV can decrease the incidence of iCCA. In terms of management, surgical resection remains the mainstay of treatment. There is a need for effective treatment modalities beyond resection in both first- and second-line treatment. In this review, we summarize the epidemiological evidence that links the two entities, discuss the pathogenesis of HBV-associated iCCA, and present the available data on the diagnosis and management of this cancer.

Key Words: Cholangiocarcinoma; Hepatitis B; Intrahepatic; Hepatocellular carcinoma; Resection; Chemotherapy

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Core Tip: Cholangiocarcinoma (CCA) is the second most common primary liver cancer. Intrahepatic CCA (iCCA), a subgroup of CCA, has a pronounced association with hepatitis B virus (HBV) infection. Recent data have strengthened this association

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both in terms of epidemiology and pathogenesis. Moreover, the potential for the development of the combined hepatocellular-cholangiocarcinoma form poses additional difficulties in the successful management of iCCA. Herein, we discuss the available epidemiological data, present the current knowledge on the pathogenesis of HBV-associated iCCA, and review all of the available information on the diagnosis and management of this aggressive cancer.

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INTRODUCTION

A 61-year-old woman with compensated liver cirrhosis due to chronic hepatitis B (CHB) presented to the outpatient clinic of our hospital for her semi-annual evaluation. She had started antiviral treatment with entecavir (0.5 mg per day) approximately 2 years prior. Her family history is significant for hepatocellular carcinoma (HCC), as her twin brother also suffered from liver cirrhosis due to CHB. On physical examination, no signs of liver decompensation were detected. Her liver synthetic function and alpha fetoprotein (aFP) value were completely normal. However, a small (1.8 cm) liver nodule with arterial enhancement was discovered in the left hepatic lobe during magnetic resonance imaging (MRI) (Figure 1). Hence, the patient underwent left hepatectomy without complications. Surprisingly, histological examination of the surgical specimen revealed a definite intrahepatic cholangiocarcinoma (CCA) (Figure 2).

This case vignette highlights the possible association between hepatitis B virus (HBV) infection and intrahepatic CCA (iCCA). iCCA is a subgroup of CCA that accounts for about 10%-20% of the total cases and classically arises above the second-order bile ducts[1]. iCCA was recently recognized as a distinct entity opposed to the extrahepatic type of CCA (eCCA)[2]. Several predisposing factors that contribute to the development of CCA have been described. A recent meta-analysis clearly showed that the degree to which these risk factors are associated with the development of this cancer significantly differ according to the tumor location[3]. Specifically for HBV infection, the odds ratio (OR) for the development of CCA is much greater for iCCA than for eCCA (4.57 *vs* 2.11). In view of these findings, several questions regarding this association arise such as how accurately can we distinguish between HCC and iCCA; what are the mechanisms that lead to the formation of iCCA; is there is a specific role for HBV in iCCA; and finally, can antiviral treatment against HBV reduce the incidence of iCCA. In this review, we summarize the epidemiological evidence that links the two entities, discuss the pathogenesis of HBV-associated iCCA, and present the available data on the diagnosis and management of this aggressive cancer.

EPIDEMIOLOGICAL STUDIES OF HBV-ASSOCIATED ICCA

To identify relevant epidemiological studies from geographically diverse regions, a comprehensive literature search of PubMed was conducted up to December 2020 without language restrictions. Studies were eligible for inclusion if they met the following criteria: were case-control or cohort study design, reported outcomes for the association between HBV infection and iCCA, and provided risk estimates with 95% confidence intervals (CIs) or sufficient data to calculate them. The following Medical Subject Headings were included: "intrahepatic cholangiocarcinoma" and "HBV." Reference lists of the retrieved articles were also manually searched to identify additional studies.

The literature search identified a total of 417 references, of which 20 case-control[4-23] and 2 cohort studies[24,25] fulfilled the inclusion criteria (Table 1). The population of the included epidemiological studies originated not only from geographical regions of high or intermediate prevalence of cholangiocarcinoma (*i.e.* Thailand, South Korea,

Table 1 Summary of epidemiological studies evaluating hepatitis B virus infection and risk of intrahepatic cholangiocarcinoma

Ref.	Study design	Region	Dates	Source	Age (yr)	Gender (% male)	Cases		Controls		OR (95%CI) for HBV infection
							Total	With exposure (%)	Total	With exposure (%)	
Petrick *et al*[4], 2017	Case-control	United States	2000-2011	Population	78.0	46.6	2092	25 (1.2)	323615	1200 (0.4%)	2.97 (1.97-4.46)
Choi *et al*[5], 2016	Case-control	United States	2000-2014	Hospital	60.6	42.9	1169	10 (0.95)	4769	8 (0.2%)	12.9 (2.69-61.61)
Huang *et al*[6], 2017	Case-control	Taiwan area of China	2003-2009	Population	67.7	52.1	4695	138 (2.9)	46942	513 (1.1%)	2.23 (1.80-2.76)
Lee *et al*[7], 2015	Case-control	South Korea	2007-2013	Hospital	67.8	50.4	83	19 (22.9)	166	9 (5.4%)	4.58 (2.00-10.50)
Chaiteerakij *et al*[8], 2013	Case-control	United States	2000-2010	Hospital	61.2	50.3	612	3 (0.5)	594	3 (0.5%)	0.97 (0.20-4.80)
Chang *et al*[9], 2013	Case-control	China	2004-2008	Population	NR	56.1	2978	257 (8.6)	11912	229 (1.9%)	4.9 (4.1-5.9)
Wu *et al*[10], 2012	Case-control	China	2002-2008	Hospital	57.9	62.1	23	12 (52.2)	52	2 (3.9%)	38.9 (7.52-201.04)
Wu *et al*[11], 2012	Case-control	China	1998-2010	Hospital	58.7	55.9	102	28 (22.5)	809	27 (3.3%)	10.52 (5.94-18.62)
Peng *et al*[12], 2011	Case-control	China	2002-2009	Hospital	53.8	60.2	98	31 (31.6)	196	25 (12.8%)	2.75 (1.27-5.95)
Welzel *et al*[13], 2011	Case-control	United States	1994-2005	Population	76.4	52.5	743	11 (1.5)	195953	442 (0.2%)	3.07 (1.43-6.58)
Tao *et al*[14], 2010	Case-control	China	1998-2008	Hospital	58.7	60.7	61	17 (27.9)	380	19 (5.0%)	7.3 (3.1-17.2)
Zhou *et al*[15], 2010	Case-control	China	2003-2006	Hospital	53.1	70.3	317	154 (48.6)	634	42 (6.6%)	9.67 (6.33-14.77)
Lee *et al*[16], 2009	Case-control	Taiwan area of China	1991-2005	Hospital	61.5	63.1	160	60 (37.5)	160	22 (13.8%)	4.99 (2.78-8.95)
Lee *et al*[17], 2008	Case-control	South Korea	2000-2004	Hospital	60.7	69.1	622	84 (13.5)	2488	125 (5.0%)	2.3 (1.6-3.3)
Zhou *et al*[18], 2008	Case-control	China	2004-2006	Hospital	53.2	66.0	312	151 (48.4)	438	42 (9.6%)	8.88 (5.97-13.19)
Shaib *et al*[19], 2007	Case-control	United States	1992-2002	Hospital	59.8	55.4	83	1 (1.2)	236	1 (0.4%)	2.9 (0.1-236.8)
Choi *et al*[20], 2006	Case-control	South Korea	2003-2004	Hospital	64.0	62.2	51	4 (7.8)	51	5 (9.8%)	0.8 (0.19-3.02)
Shaib *et al*[21], 2005	Case-control	United States	1993-1999	Population	78.7	51.7	625	1 (0.2)	90834	182 (0.2%)	0.8 (0.1-5.9)
Yamamoto *et al*[22], 2004	Case-control	Japan	1991-2002	Hospital	64.6	58.0	50	2 (4.0)	205	5 (2.5%)	1.84 (0.34-10.11)
Donato *et al*[23], 2001	Case-control	Italy	1995-2000	Hospital	65.0	80.8	23	3 (13.0)	824	45 (5.5%)	2.7 (0.4-18.5)
Tanaka *et al*[24], 2010	Cohort	Japan	1991-1993	Population	40-60	54.4	9.08/100000 p-yrs		0.66/100000 p-yrs		8.56 (1.33-55.20)
Fwu *et al*[25], 2011	Cohort	Taiwan area of China	1983-2000	Population	28.29	0	0.43/100000 p-yrs		0.09/100000 p-yrs		4.80 (1.88-12.20)

Hepatitis B virus infection status was identified by positive serum Hepatitis B surface antigen (HBsAg), unless reported otherwise. Cohort studies reported incidence rates of intrahepatic cholangiocarcinoma per 100000 HBsAg seropositive person-years (p-yrs). CI: Confidence interval; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; iCCA: Intrahepatic cholangiocarcinoma; NR: Not reported; OR: Odds ratio.

Japan, Mainland China and Taiwan area of China) but also from regions of low preva

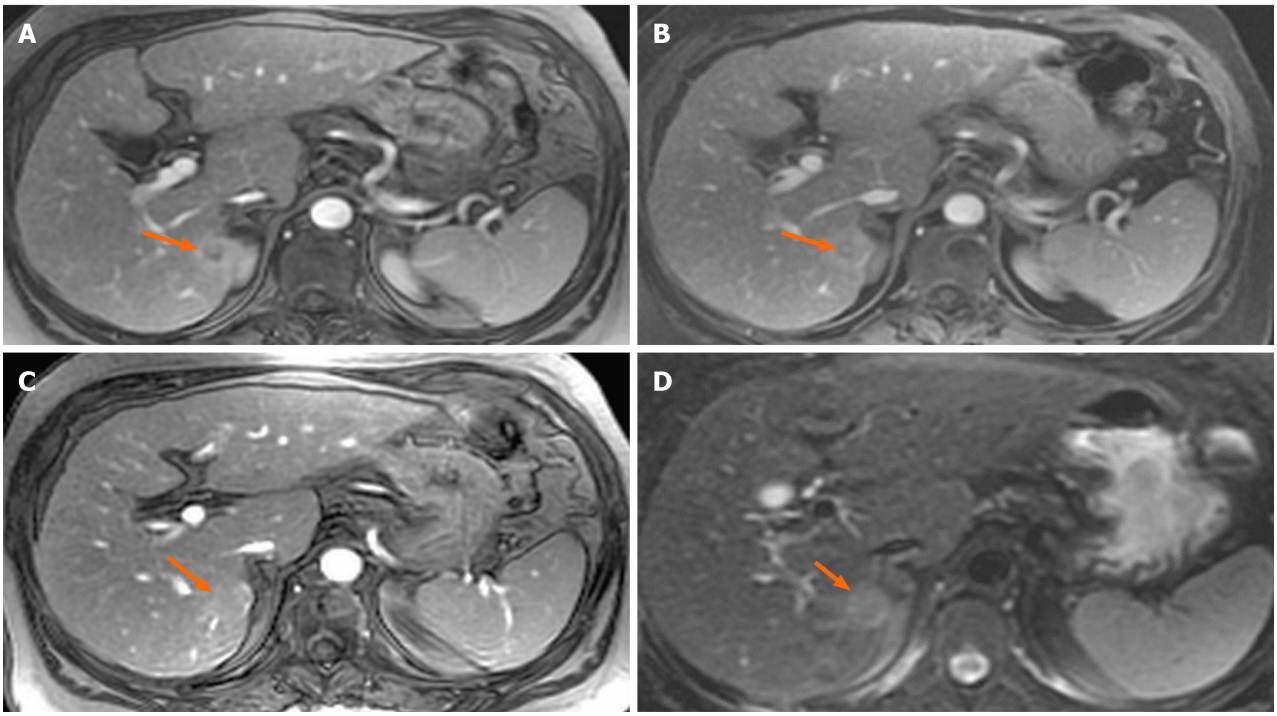


Figure 1 A small (1.8 cm) liver nodule with arterial enhancement is discovered in the left hepatic lobe during magnetic resonance imaging. A: T1, arterial phase; B: T1 portal phase; C: T1 delayed phase; D: T2.

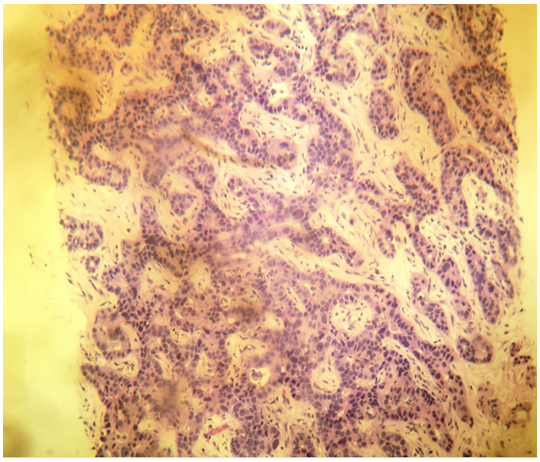


Figure 2 Histological findings typical of cholangiocarcinoma.

Twenty case-control studies were included (seven from Mainland China[9-12,14,15,18], six from the United States[4,5,8,13,19,21], three from South Korea[7,17,20], two from Taiwan area of China[6,16] and one each from Japan[22] and Italy[23]), involving 14899 patients and 681258 cases. Notably, only 4 of the 20 retrieved studies were published in the last 5 years. Population-based controls were employed in 3 studies from the United States[4,13,21] and 1 from Mainland China[9] and the Taiwan area of China[6], whereas the remaining 15 studies were hospital-based. HBV infection status was defined by the presence of hepatitis B surface antigen (HBsAg) in 15 studies[5,7,8,10-12,14-20,22,23] and by international classification of diseases-9 coding in 5 studies[4,6,9,13,21]. Seropositivity of hepatitis B core antibody (HBcAb) was assessed in five studies[10-12,14,19]. All of the included case-control studies reported adjustments for age and gender. Fourteen studies showed a statistically significant association between HBV infection and risk of iCCA (range of individual OR = 2.23-12.9)[4-7,9-18], whereas the rest of the studies (n = 6) did not[8,19-23]. These six studies were concluded prior to 2002 in geographically different areas. Geographical variations in iCCA incidence may be explained by differences in environmental and genetic risk factors. Moreover,

observational studies are difficult to control for confounders, which could present an important risk factor and influence the prognostic value of HBV infection in iCCA.

Two large-scale cohort studies with a total of 1937215 participants evaluating the correlation between HBV infection and iCCA were identified (Table 1). A retrospective cohort study from Japan involved 154814 blood donors with a median follow-up period of 7.6 years and reported an iCCA incidence rate of 0.88 per 100000 person-years[24]. Compared with individuals negative for both HBsAg and hepatitis C virus antibody (anti-HCV), those who tested positive for HBsAg had a significantly higher risk of iCCA (hazard ratio [HR] = 8.56; 95%CI: 1.33-52.20). The latter study from Taiwan area of China included a cohort of 1782401 pregnant women with a median follow-up of 6.91 years and reported 18 cases of iCCA[25]. Women seropositive for HBsAg had significantly increased incidence rates of iCCA (0.43 per 100000 person-years) than HBsAg-negative women (0.09 per 100000 person-years) with an age-adjusted hazard ratio HR = 4.80; 95%CI: 1.88-12.20.

Occult HBV infection (OBI), which is defined by the detection of HBV DNA in the serum and liver tissue of HBsAg-negative individuals with or without serological markers of previous viral exposure[28], is an emerging risk factor for iCCA. HBV maintains its pro-oncogenic properties in patients with OBI[29]. Indeed, epidemiological and molecular studies have indicated that OBI represents an important risk factor for the development of cirrhosis and HCC[30,31]. Relevant evidence from pathological studies, investigating the presence of HBV DNA, genes, and proteins in iCCA tissue specimens, also suggests a possible etiological role of OBI in iCCA[32-34]. A case-control study of 183 cryptogenic iCCA patients and 549 healthy individuals confirmed that HBsAg seroclearance does not signify eradication of HBV and may not entirely prevent the development of iCCA in Chinese patients[32]. Pollicino *et al*[33] investigated the presence of HBV DNA in liver tissue specimens from 47 HBsAg-negative patients with iCCA and 41 paired non-tumor liver tissues. A high prevalence of OBI in iCCA patients, along with the presence of both free viral genomes and integrated HBV DNA in the tumor tissue, suggested an involvement of HBV in the carcinogenesis of iCCA not only in overt but also in occult infection[33].

To date, there are limited data on the relationship between iCCA and HBV genotype. Barusrux *et al*[35] investigated the prevalence of HBV and HCV infection among 295 CCA patients in northeast Thailand[35]. In this study, HBV genotypes C (73.3%) and B (26.7%) were detected, in line with the HBV genotype distribution in Thailand[36]. In a cohort of 11 cases of iCCA in the eastern United States, liver specimens were examined for HBV DNA and HCV RNA and the genotypes observed represented the dominant genotype (genotype A) in this area[37]. Currently, there is no sufficient evidence to support an etiological association between specific HBV genotypes and iCCA.

Meta-analyses of HBV-associated iCCA

To address the inconsistent results reported in previous epidemiological studies, an overview of the most comprehensive meta-analyses exploring the association between HBV infection and iCCA is provided (Table 2). A total of six systematic reviews and meta-analyses were retrieved[3,38-42], two of which focused on the risk factors of iCCA alone[41,42]. The remaining studies assessed HBV infection as a sole factor or among other risk factors for both iCCA and eCCA[3,38-40]. Of these, two meta-analyses were published in 2020 (one from the United Kingdom[3] and the other from China[38]), one in 2016 from China[39] and three in 2012 (two from China[40,42] and one from the United States[41]). In four meta-analyses, the population of the primary epidemiological studies originated from both Eastern and Western countries[3,40-42], whereas the remaining two meta-analyses included studies only from Asia[38,39]. The period of data collection among these primary studies ranged from 1987 to 2014.

All of the included meta-analyses suggested that HBV infection was significantly associated with an increased risk of iCCA (range of pooled OR = 3.17-5.98)[3,38-42]. A moderate to severe degree of statistical heterogeneity was noted across the primary epidemiological studies, as assessed by Cochran's Q and I^2 statistic (Table 2). Publication bias was identified in two meta-analyses[38,42].

To the best of our knowledge, the most up-to-date systematic review and meta-analysis were performed by Clements *et al*[3] in 2020. A total of 13 risk factors, including HBV, were evaluated for iCCA and eCCA. Eighteen case-control studies were included to study the association between HBV and iCCA. The pooled risk estimate (OR = 4.57; 95%CI: 3.43-6.09) showed a statistically significant increased risk of iCCA with HBV infection. Furthermore, meta-regression analysis indicated that in Eastern countries, HBV conferred a greater risk of iCCA than in Western countries. Similar geographical differences for the association of HBV with iCCA have been

Table 2 Summary of meta-analyses evaluating hepatitis B virus infection as a risk factor for intrahepatic cholangiocarcinoma

Ref.	Number of included studies	Data collection period	Regions	Cases	Controls	Pooled OR (95%CI) for HBV infection	Heterogeneity	Publication bias
Clements *et al*[3], 2020	Case-control = 18	1991-2014	Eastern = 12. Western = 6	14825	681181	4.57 (3.43-6.09)	I^2 = 83% Q = 100.88 (P < 0.001)	Funnel plot: Symmetrical. No bias
Tan *et al*[38], 2020	Case-control = 6	1998-2013	Eastern = 6	3623	13597	5.98 (4.08-8.76)	I^2 = 69% Q = 16.01 (P = 0.07)	Funnel plot: Relatively symmetrical. Moderate bias
Zhang *et al* [39], 2016	Case-control = 11	1991-2013	Eastern = 11	4966	17752	3.18 (2.35-4.30)	I^2 = 84% Q: P < 0.001	Begg's test: P = 0.75, Egger's test: P = 0.42. No bias
Li *et al*[40], 2012	Case-control = 11. Cohort = 2	1991-2008	Eastern = 10. Western = 3	2402. NA	96266. NA	3.42 (2.46-4.74)	I^2 = 75% Q = 48.37 (P < 0.001)	Funnel plot: Symmetrical Begg's test: P = 0.70, Egger's test: P = 0.26. No bias
Palmer *et al* [41], 2012	Case-control = 8	1991-2008	Eastern = 5. Western = 3	2753	291756	5.54 (3.19-9.63)	I^2 = 86.3% Q = 51.2 (P < 0.001)	Funnel plot: Symmetrical. No bias
Zhou *et al* [42], 2012	Case-control = 12. Cohort: = 2	1987-2009	Eastern = 11. Western = 3	2362. NA	96452. NA	3.17 (1.88-5.34)	I^2 = 81% Q: P < 0.001	Funnel plot: Asymmetry. Moderate bias

Statistical heterogeneity was assessed by the Cochran's Q statistic and I^2 statistic. Significant heterogeneity is considered if Q: P < 0.10 or I^2 > 60%. Publication bias was evaluated using the Begg's funnel plot and Egger's test with P < 0.10 as an indication of publication bias. CI: Confidence interval; HBV: Hepatitis B virus; ICC: Intrahepatic cholangiocarcinoma; NA: Not applicable; OR: Odds ratio.

reported by Li *et al*[40], however Palmer and Patel[41] did not reveal any significant difference between Eastern and Western populations.

PATHOGENESIS OF HBV-ASSOCIATED ICCA

Cholangiocarcinoma is a multifactorial heterogeneous disease of diverse predisposing factors. However, regardless of underlying etiology, chronic inflammation seems to be a common denominator of most risk factors for CCA as they are usually associated with inflammation or cholestasis. Chronic inflammation leads to increased production of a multitude of inflammatory mediators such as interleukin-6 (IL-6), tumor necrosis factor (TNF), cyclooxygenase-2, and Wnt, resulting in an "activated" environment favoring successive mutations of tumor suppressor genes, and other oncogenes. Other mediators found to be upregulated in CCA include transforming growth factor beta (TGF-β), vascular endothelial growth factor, hepatocyte growth factor, and several microRNAs[43]. Most of those changes promote angiogenesis and cell proliferation.

In particular, liver cirrhosis regardless of etiology represents an inflammatory process that is clearly associated with the development of iCCA. Several population-based studies have confirmed cirrhosis as an important risk factor not only for HCC but also for iCCA[44]. A meta-analysis found that cirrhosis causes iCCA with an OR as high as 22[41]. Furthermore, a retrospective analysis of United States-based Surveillance, Epidemiology, and End Results found that cirrhosis is associated with both iCCA (OR 8.26) and eCCA (OR 3.83)[4]. As chronic infection with HBV and HCV account for most cases of cirrhosis globally, it is not surprising that a definite association between viral hepatitis infection and iCCA has been observed. A large metanalysis produced an OR for iCCA of 3.17 and 3.42 for HBV and HCV, respectively [42]. A distinct phenotype of viral hepatitis-related iCCA that more frequently affects younger males and is characterized by mass-forming tumors with cholangiolar differentiation, higher aFP, lower carbohydrate antigen (CA) 19-9 levels and higher N-cadherin expression has been suggested[12,45,46]. Such tumors are more commonly associated with cirrhosis or elevated prothrombin time. Recent studies have suggested that HBV-associated iCCA displays infrequent nodal metastases and confers better a prognosis, whereas patients with CCA associated with HCV may fare worse[47,48]. In addition, viral hepatitis-associated CCA more frequently expresses programmed death-ligand 1 (PD-L1), which in theory may be exploited for the development of immunotherapy strategies[49].

Inflammation is a multifactorial and diffuse process. It involves multiple pathways acting through diverse mechanisms in an environment conducive of proliferation and oncogene mutations leading to malignant transformation. As a result, there has been no single molecular alteration characteristic of CCA. IL-6, a strong proinflammatory cytokine has a pleiotropic role, by increasing cell proliferation through activation of the Janus kinase-signal transducer and activator of transcription pathway; it also promotes expression of antiapoptotic myeloid leukemia cell differentiation protein and the activation of both the Akt and mitogen-activated protein kinase kinase (MEK)-extracellular signal-related kinase (ERK) pathways, which results in strong proliferative and anti-apoptotic effects[50-52]. In such an activated environment, increased DNA instability may lead to critical genetic alterations of the excited cells, and also increases the probability of mutations of important genes such as tumor suppressor gene p53 and the MYC proto-oncogene, among others[53]. The DNA instability is at least partially attributable to the effect of TNF-α, which among others, causes the upregulation of activation-induced cytidine deaminase, an enzyme that can create DNA mutations by converting cytosine to uracil.

In addition to the inflammatory changes caused in the liver tissues by the continuous presence of the virus, it seems that hepatitis viral components may act as oncogenes and promote oncogenicity *per se*. In particular, hepatitis B x (HBx) protein has been implicated in carcinogenesis. Experiments with transgenic mice have demonstrated that its expression enhances IL-6-dependent proinflammatory effects and causes malignant transformation towards both HCC and CCA[54].

The presence of HBV genes in the genome of CCA has been described. Although the exact effect on tumorigenesis is unknown, the observed proximity of the insertion sites to the gene encoding the tumor suppression p53 is intriguing. Wang *et al*[55] using *in situ* hybridization demonstrated the presence of HBV genes in 33 of 40 iCCA cases tested; the most common gene found was again HBx[55]. In a study by Pollicino *et al* [33] employing a PCR technique, incorporation of HBV genes in patients with iCCA and no overt viral infection was detected in tumor or non-tumorous liver cells in 29 of the 47 examined cases, suggesting the importance of occult viral infection in the development of CCA. Most cases involved the HBx gene.

Expression of the culprit HBx gene may be documented by immunohistochemistry in about 70% of patients with iCCA and correlates with peripheral tumors and higher aFP levels, but the exact percentage obviously depends on the epidemiology of the tested population[56]. Co-expression of HBx and HCV core proteins in transgenic zebra fish livers induces fibrosis and iCCA[57], and is associated with activation of the phosphorylated Smad3L oncogenic pathway with consequent phosphorylation and activation of MEK-ERK proteins. Bile duct proliferation, fibrosis, and iCCA were markedly reduced by knockdown of TGF-β, suggesting the important role of this mediator. Semaphorine 3B is a recently identified tumor suppressor gene encoded on chromosome 3p21.3 and is connected to p53 activity. Silencing of the locus due to gene or promoter hypermethylation or loss of homozygosity was detected in several CCA tumor cells, and as expected, was associated with the downregulation of mRNA transcripts[43].

These dysregulations result in a broad variety of somatic mutations that have been recently identified using next-generation sequencing, some of which impair the function of known oncogenes. Almost any gene implicated in carcinogenesis is abnormal in CCA, attesting to the heterogeneity of the disease and the lack of a pathognomonic driver mutation[58]. Thus, KRAS mutations may affect 20% of CCA. Mutations of isocitrate dehydrogenase 1 (IDH1) and IDH2 genes occur in approximate 20% of iCCA. IDH1 and IDH2 genes encode for enzymes active in the citric acid cycle (Krebbs cycle), and their loss of function mutations results in accumulation of an abnormal oncometabolite R-2-hydroxyglutarate which interferes with the function of several pathways and causes epigenetic DNA changes. Other alterations of iCCA include fibroblast growth factor receptor 2 (FGFR2) mutations. Translocation or amplification of the FGFR2 locus is found in 19% of 122 iCCA patients[59]. Tumors of such patients tend to be of the mass-forming type and compared to non-FGFR2 altered tumors, they secrete less mucus and are more commonly associated with HBV infection.

Although some of these alterations appear to be important for the malignant behavior of the tumor, they may not represent the initiating transforming event, which should be sought earlier in the pathogenesis of iCCA. There is enough evidence to suspect that chronic, overt or occult, infection with hepatitis virus may trigger the transformation of infected cells in the context of a conducive, inflammatory environment. Of the various HBV genes, evidence suggests that HBx plays the most crucial role. HBx protein promotes cell cycle progression, inactivates negative growth

regulators, and binds to and inhibits expression of the p53 tumor suppressor gene, other tumor suppressor genes, and senescence-related factors[60]. It appears that some of these actions are mediated through overexpression of telomerase (hTERT) mainly by interfering with its promoter. Thus, the importance of HBx was demonstrated in an experiment involving HCC cell lines, whereby transfection with HBx resulted in the overexpression of hTERT a factor important for the non-senescence of transformed cells[61]. Using current sequencing technology, it appears that the integration of viral sequences to human DNA tends to aggregate in the vicinity of the telomerase gene, although other sites of viral gene insertion of unclear significance have been observed [62]. Interestingly, HBV gene insertions (hepatitis B S and/or C gene fragments) have also been noted in the normal hepatocytes of patients, albeit at reduced frequency. The capacity of HBx gene insertion to directly promote the expression of TERT was demonstrated in a direct transfection experiment in CCA cell lines; HBx gene resulted in cis-activation of TERT mRNA transcription[46]. Figure 3 summarizes the potential pathological mechanisms that lead to carcinogenesis in patients with hepatitis B.

It can be concluded that mounting evidence supports the theory that chronic, overt, or latent infection with HBV has direct causative effects on the development not only of HCC but also of iCCA, perhaps by acting on a common progenitor cell. The effect of certain viral genes, such as HBx, seems to have direct oncogenic capacity beyond the genetic instability produced by the chronic inflammation, perhaps aided by the latter. These observations support the epidemiologic data linking CHB to the development of iCCA and call for further studies to fully reveal the intricate interaction of the virus with the cellular mechanisms leading to carcinogenesis.

CLINICAL PRESENTATION

Patients with iCCA usually present with unspecific symptoms such as abdominal pain, malaise, night sweats, and cachexia[63]. Jaundice is usually a sign of advanced disease. In particular, patients with HBV-associated iCCA can be diagnosed before they develop any symptoms, due to the fact that they undergo annual or semi-annual surveillance for HCC, which enables iCCA diagnosis at a relatively early stage[64].

DIAGNOSIS

Imaging techniques

Imaging modalities, such as computed tomography (CT), MRI, and fluorodeoxy-glucose positron emission tomography (FDG-PET) can be of great value in the management of iCCA in terms not only of diagnosis but also of staging, follow-up, and assessment of treatment response.

CT scan serves as the standard imaging method for the preoperative assessment of iCCA. It provides a comprehensive evaluation of the primary tumor, the relationship between the primary tumor and adjacent structures such as the hepatic artery or portal vein, and the whole abdomen surveillance for potential metastases[65]. On both contrast-enhanced CT and MRI scans, iCCA usually presents as a mass with irregular borders, peripheral rim enhancement on the arterial phase with progressive homogeneous contrast agent uptake until the delayed or stable uptake during late dynamic phases[66,67]. However, smaller tumors are less likely to demonstrate these characteristics and may be hard to distinguish from HCC, especially in the context of chronic liver diseases.

The utility of FDG-PET for iCCA diagnosis and management is still controversial. Although mass-forming iCCA can be detected with a reported sensitivity of 85% to 95%, it cannot be used for the diagnosis of iCCA as it is not specific enough[68]. However, it could prove useful in detecting distal, unexpected metastases. In fact, a recent meta-analysis suggested that it can bring changes to the management of a substantial portion of iCCA patients[69]. Therefore, it could be considered an option prior to surgical resection (SR) to rule out distant metastatic foci.

Serum markers

Tumor markers in serum are not specific for iCCA, but may be of diagnostic value in some cases. Widely used tumor markers such as CA 19-9 and carcinoembryonic antigen have significant overlap with other cholestatic diseases and low sensitivity for early stage disease, consequently limiting their use[70]. However, high values of these

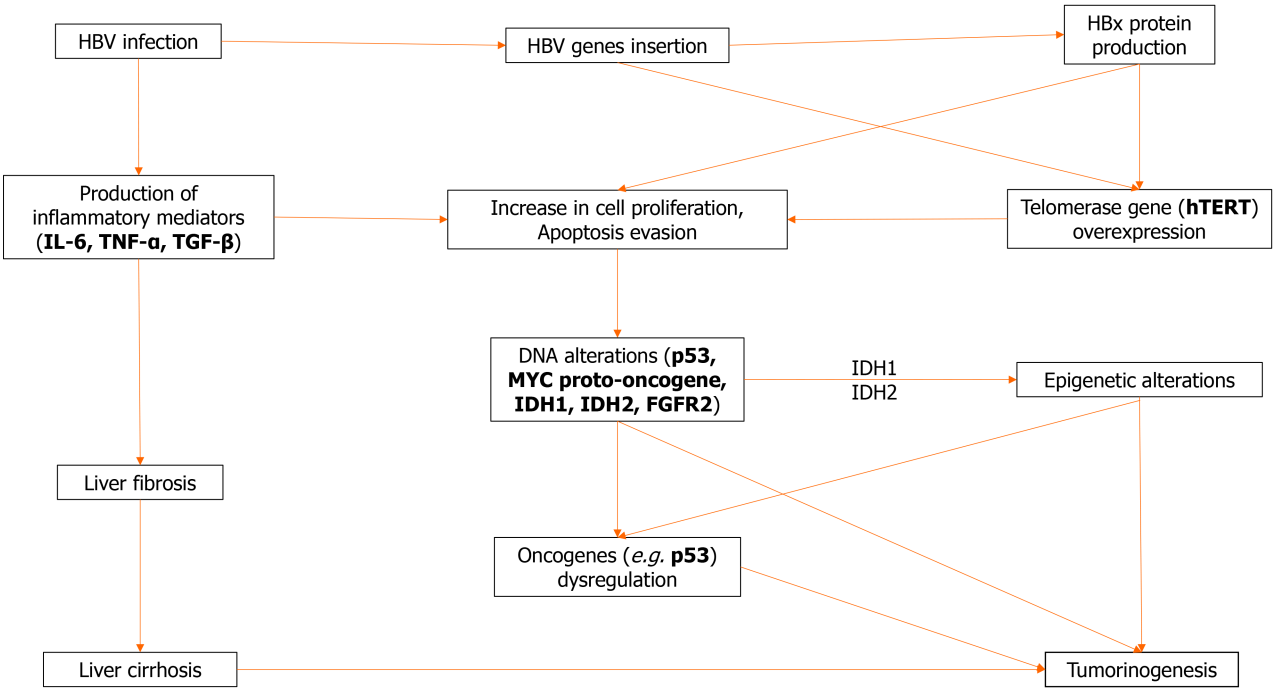


Figure 3 Pathological mechanisms that lead to tumorigenesis in patients with hepatitis B virus infection. FGFR: Fibroblast growth factor receptor; HBV: Hepatitis B virus; IDH: Isocitrate dehydrogenase; IL-6: Interleukin 6; TGF-β: Transforming growth factor β; TNF-α: Tumor necrosis factor α.

markers have been connected with a deterioration in overall survival (OS)[71,72]. As a result, high (> 500 ng/mL) levels of CA 19-9 in the absence of jaundice are considered a relative contraindication for resection of iCCA[73].

However, in cases of HBV-associated iCCA, these markers tend to be even less sensitive[48]. Instead, it has been suggested that such patients tend to have higher levels of aFP, a serum marker widely used for HCC diagnosis, probably because of the fact that both malignancies originate from hepatic progenitor cells, which have the ability to produce aFP[16,46,74,75].

Histological diagnosis

Pathological diagnosis is recommended for all patients who will undergo systemic chemotherapy or radiation therapy, or take part in a therapeutic clinical trial[68,76]. It should be noted that although a positive result demonstrates the presence of the tumor, a negative result does not exclude it because of the potential sampling errors. In such cases, repeat sampling is often required to confirm the correct diagnosis[77].

According to the World Health Organization (WHO) classification for biliary tract cancer[78], iCCA can be further classified to two main subtypes according to the level or size of the affected duct; large duct type, which resembles eCCA and small duct type, which shares etiological, pathogenetic, and imaging characteristics with HCC and usually develops on a background of chronic liver disease (such as chronic viral hepatitis and cirrhosis)[58]. Small bile duct iCCA presents as a tubular or acinar adenocarcinoma with nodular growth invading the liver parenchyma, and with no or minimal mucin production[79-81]. Characteristic findings of small bile duct iCCA are IDH1 and IDH2 mutations or FGFR2 fusions or translocations[80-82]. In HBV-associated iCCA, there are data indicating the presence of hyperactivated programmed cell death protein 1/PD-L1 signaling and increased expression of N-cadherin and HBx protein[45,49,56]. It remains to be seen if these diagnostic characteristics can also serve as potential therapeutic targets.

Staging

iCCA is mainly staged according to the 8th edition of the American Joint Committee on Cancer (AJCC) TNM system (Tables 3 and 4). Despite providing some meaningful contribution to the patient's prognostication, it does have some rather important limitations. First, although the tumor size was introduced for the first time in the latest AJCC Cancer Staging Manual, the only discriminating size cut-off used is the one of < 5 cm encompassing all T1 tumors. However, there are some data indicating that patients with very small (< 2 cm) tumors have potentially better outcomes in terms of

Table 3 American Joint Committee on Cancer staging classification for intrahepatic cholangiocarcinoma (8th edition, 2017)

Staging classification	
T1a	Solitary tumor ≤ 5 cm without vascular invasion
T1b	Solitary tumor > 5 cm without vascular invasion
T2	Solitary tumor with vascular invasion, or multiple tumors with or without vascular invasion
T3	Tumor perforating the visceral peritoneum
T4	Tumor involving the local extrahepatic structures by direct invasion
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis present
M0	No distant metastasis
M1	Distant metastasis present

Table 4 American Joint Committee on Cancer staging classification for intrahepatic cholangiocarcinoma (8th edition, 2017)

Stage	T	N	M
Ia	T1a	N0	M0
Ib	T1b	N0	M0
II	T2	N0	M0
IIIa	T3	N0	M0
IIIb	T4	N0	M0
	Any T	N1	M0
IV	Any T	Any N	M1

survival, rendering them eligible for different therapeutic modalities[83]. In addition, the latest TNM system fails to include parameters such as performance status (PS) or the severity of liver function impairment. Both of these limitations are clearly extremely important for assessing the prognosis of patients with HBV-associated iCCA, as very small tumors can be detected during surveillance programs and may also have advanced parenchymal liver disease.

THE CASE OF COMBINED HCC AND CHOLANGIOCARCINOMA

Combined HCC-CCA (cHCC-CCA) is a rare type of primary liver cancer (PLC), which has been defined as a distinct carcinoma displaying features of both hepatocytic and cholangiocytic differentiation[84]. Since the first description of this entity in 1949[85], significant terminology changes have been introduced. The 2010 WHO classification system distinguishes a classical form of cHCC-CCA and three variants with stem/progenitor cell features including typical, intermediate cell, and cholangiolocellular subtypes[86]. However, molecular advancements throughout the years have challenged the validity of stemness; thus, the term "typical subtype" has been abandoned; cholangiolocellular carcinoma is currently considered to be a subtype of iCCA, but it can still be within the spectrum of cHCC-CCA only if an HCC component is also involved and is currently addressed as "cholangiolocarcinoma"; PLCs consisting exclusively of intermediate cells are specified as intermediate cell carcinomas, while the role of intermediate cells in cHCC-CCA is still debated[84,87,88].

Large series have reported that cHCC-CCA represents 0.77%-5.8% of all PLCs[89,90]. Viral hepatitis (HBV, HCV), alcohol abuse, and cirrhosis have been associated with this cancer. Notably, cirrhosis and HBV infection are more commonly related with cHCC-CCA than iCCA, although with lower rates compared to HCC[91-94]. Most patients are aged between 50 and 60 years, with comparative studies claiming presentation in a younger age than iCCA[91,95], while others have not managed to point out significant age differences[92,93]. Male predominance is well documented

(65%-80%)[89,90,95,96] and tends to be more notable opposed to iCCA patients[92]. Studies focusing on HBV-related cHCC-CCA present an even greater male to female ratio that reaches 9:1[97,98].

Imaging features may resemble patterns of HCC, CCA, or both, therefore rendering radiological modalities insufficient to ensure definite diagnosis. Arterial phase hyperenhancement and portal/delayed phase hypoenhancement (washout) are characteristic findings of HCC in CT, MRI, and contrast-enhanced ultrasound; however, they could also be displayed by cHCC-CCA lesions and lead to misdiagnosis [93,99]. Similarly, cHCC-CCA tumors can mimic CCA by displaying gradual enhancement and peripheral irregular rim-like contour. According to Wang *et al*[92], in the former case the presence of lymphadenopathy, elevation of CA 19-9 and/or biliary dilation are in favor of cHCC-CCA, whereas the presence of pseudocapsule and well-defined margin are more suggestive of HCC. In the latter case when cHCC-CCA resembles features of CCA, the presence of simultaneous cirrhosis, HBsAg positivity, pseudocapsule and/or portal vein thrombosis should raise suspicion of cHCC-CCA. Furthermore, concurrent elevation of both aFP and CA 19-9 or discordance between tumor markers and imaging features (*i.e.* radiological hallmarks of HCC accompanied by increased CA 19-9 or lesions resembling CCA with raised aFP) should prompt consideration of cHCC-CCA diagnosis[100,101]. Histopathological examination of biopsies or surgical specimens based on routine stains ultimately establishes the correct diagnosis. Immunohistochemical markers can provide additional information, but are of secondary importance[84].

Complete excision with negative margins and sufficient residual liver volume is the goal of treatment[102]. In a large series of 390 HBV-related cHCC-CCA, the median OS after SR was 20 mo and negative prognostic factors included male gender, number of tumors ≥ 2, major thrombus, gamma-glutamyl transferase (GGT) > 60 IU/L, and CA 19-9 levels[98]. In a recent systematic review, data analyses of 14 studies showed that patients with cHCC-CCA, HCC, and CCA undergoing SR had 5-year survival rates of 32.7%, 47.5%, and 30.3% respectively[96]. Similar outcomes have been published about liver transplantation (LT): HCC patients achieve considerably greater survival rates than cHCC-CCA and CCA subgroups[95,103]. The role of locoregional treatment and systematic chemotherapy in the management of cHCC-CCA remains uncertain. A retrospective analysis of 50 patients with unresectable cHCC-CCA undergoing transarterial chemoembolization (TACE) demonstrated that better response rates could be attained when careful selection is applied (*i.e.* hypovascular tumors respond poorly due to inability of chemotherapeutic agents to reach target)[94]. Limited data about chemotherapy are available; however, it is noteworthy to mention the results of two recent multicenter studies using platinum-based regimens, which were associated with favorable outcomes compared to sorafenib monotherapy[104] and could be potentially effective when used together with gemcitabine as first-line treatment for advanced, unresectable cHCC-CCA[105].

TREATMENT OF ICCA

SR

SR is the treatment of choice for all iCCA patients, since it is the only established therapy capable of achieving a possible cure[68,76]. Conventional surgical aim of a SR should be complete tumor resection with negative microscopic (R0) margins while preserving adequate liver volume to avoid post-operative liver failure[73].

The eligibility of a patient for curative surgery should always be determined by a multidisciplinary board on a case-by-case basis. Given the poor prognosis of patients who have multifocal disease or clinically evident lymph node metastasis, SR should be precluded for these patients[106,107]. Regarding macroscopic vascular invasion, although it should generally be considered a contraindication, there are some data from specialized centers supporting that major vascular resection is not associated with worse perioperative or oncologic outcomes[108]. Obviously, as with HCC[109], liver function should be assessed in patients with underlying liver cirrhosis. Restrictions for resection are recommended in cases where liver function is significantly impaired and/or the patient's PS is poor.

As aforementioned, patients with HBV-associated iCCA are often diagnosed at a relatively early stage. This is probably the reason why the proportion of iCCA patients who undergo curative resection is significantly higher for HBV-positive than HBV-negative patients[110]. HBV infection has been identified by many studies as an independent factor associated with favorable outcomes, suggesting that better

prognosis of HBV associated iCCA may be perhaps explained by less aggressive biological nature[111-115]. In fact, a meta-analysis showed that lymph node metastasis occurred less commonly in HBV-associated iCCA than iCCA of other etiology (OR = 0.39)[48]. In a large single-center retrospective study from China that included 1333 iCCA patients (608 HBsAg-positive and 725 HBsAg-negative) the median post-operative survival was 19 mo for HBsAg(+) compared to 12 mo for HBsAg(-) patients ($P < 0.001$)[116]. Factors that have been associated with better outcomes for patients with HBV-associated iCCA include tumor diameter < 5 cm, better tumor differentiation, lower serum levels of aFP or CA 19-9 and absence of vascular invasion, lymph node metastasis, extrahepatic metastasis or TP53 mutation[113,117,118].

Adjuvant chemotherapy

Until a few years ago, the role of adjuvant chemotherapy in the setting of iCCA was not well defined because of a complete lack of data from randomized trials. However, a meta-analysis of retrospective cohorts indicated that administration of chemotherapy in the adjuvant setting provides a survival benefit to patients with iCCA[119]. This contrasted with the randomized PRODIGE 12[120] trial (gemcitabine and oxaliplatin [GEMOX] *vs* surveillance for patients with resected biliary tract cancer) which failed to produce statistically significant results. On the other hand, the BILCAP trial (capecitabine *vs* observation for patients with resected biliary tract cancer)[121], showed a benefit in terms of recurrence-free survival in the intention-to-treat analysis (HR = 0.75, $P < 0.05$) and also in terms of OS in the pre-planned sensitivity analysis (HR = 0.75, $P < 0.05$), both in the first 24 mo from randomization. However, no statistically significant benefit was observed in the intention-to-treat OS analysis of the whole population or the iCCA subgroup. Based on these partial benefits, the American Society of Clinical Oncology published a new guideline recommending that all patients undergoing SR of any biliary tract tumors should be offered adjuvant capecitabine chemotherapy for 6 mo[122]. More information is expected in the foreseeable future from the ACTICCA-1 study (NCT02170090), a phase III trial investigating the clinical performance of the GEMOX combination compared to capecitabine in the adjuvant setting.

Antiviral treatment

There are not much data concerning the role of antiviral treatment in the management of HBV-associated iCCA. However, one retrospective study demonstrated that antiviral treatment, even when initiated post-operatively, can provide a significant benefit to the patients compared to patients with high (> 2000 IU/mL) HBV-DNA levels who are left untreated (5-year recurrence rate 70.5% *vs* 86.5%, $P < 0.001$ and 5-year OS rate 43% *vs* 20.5%, $P < 0.001$ respectively)[123].

In 2017, a large-scale retrospective study from Taiwan area of China that was based on data from the National Health Insurance Research Database showed that treatment with nucleos(t)ide analogues (NA) is an independent factor for the development of HBV-associated iCCA (HR 0.44, $P = 0.004$)[124]. In the population treated with NA, the 5-year cumulative incidence of iCCA was lower compared to the NA-untreated group (0.17% *vs* 0.39%, $P = 0.005$), stating that treatment with NA could contribute in preventing HBV-associated iCCA, in a similar way as it contributes to reducing the risk of HBV-associated HCC.

LT

LT for iCCA is still a controversial issue. In the era of organ shortage, iCCA was traditionally considered a contraindication for LT, as both the OS and disease-free survival were disappointing. It should be noted that most of this information came from small, retrospective studies with much heterogeneity in transplant protocols. In certain studies, the 5-year OS rate ranged between 30% to 60%[125-128].

Studies focusing on favorable groups of patients who could potentially derive significant benefit from LT have been reported. In a multicenter retrospective study from Spain[129], a subgroup of patients ($n = 8$) with "very early" iCCA (single tumor, < 2 cm) was highlighted. The 5-year OS in this subgroup was 73% compared to 35% for the patients with single tumors > 2 cm or multinodular tumors ($n = 21$). Another international, multicenter study showed similar results, as the actuarial survival rate for "very early" iCCA was 65%[83]. A recent meta-analysis confirmed the results of such efforts (5-year pooled OS rate 71% *vs* 48% for advanced iCCA), stating that a solitary < 2 cm iCCA in a background of cirrhosis should not preclude consideration of LT[130]. It should be noted though, that the application of LT for cirrhotic patients, with "very early" iCCA unresectable due to impaired liver function still requires

validation by a prospective study. Such a study (NCT02878473) is currently recruiting patients and is expected to provide primary results by 2026.

Another interesting subgroup of iCCA patients, who could substantially benefit from LT, are those with locally advanced, unresectable (due to tumor size, location, or multifocality) disease with a durable response or stability after neoadjuvant chemotherapy[131]. In fact, in an interim analysis of a small series of patients (n = 6), with large, unresectable iCCA subjected to LT after an at least 6-mo disease stability under neoadjuvant chemotherapy with gemcitabine-based regimens, the 5-year survival was 83%, although, of note, 5-year recurrence rate was 50%[132].

In light of this information, a recent consensus approved by the International Liver Transplant Society recommends that patients with "very early" iCCA may benefit from upfront LT, whereas those with advanced, unresectable iCCA in a non-cirrhotic state may become LT candidates if the disease remains stable after neoadjuvant therapy[133].

MANAGEMENT OF UNRESECTABLE DISEASE

Unfortunately, many patients with iCCA present with advanced disease that is not amenable to surgery. When assessing a patient with unresectable iCCA, the medical team managing his care should first take into consideration the patient's PS, as determined by the Eastern Cooperative Oncology Group PS (ECOG-PS) scale. Patients with ECOG-PS > 2 are unlikely to benefit from any treatment and should be managed with best supportive care. After that, the stage of the disease and the molecular profile of the tumor should be assessed in order to decide the most suitable therapeutic approach for each patient.

Systemic chemotherapy

Systemic chemotherapy is generally recommended for patients with ECOG-PS 0-1[68]. The combination of gemcitabine and cisplatin (GEMCIS) is used as the first-line therapeutic regimen in patients with advanced iCCA[105,134]. In the ABC-02 trial (n = 410, all biliary tract cancers) the researchers observed a statistically significant difference in OS and in progression-free survival (11.7 mo *vs* 8.1 mo and 8 mo *vs* 5 mo, respectively) in favor of the GEMCIS combination compared to gemcitabine alone. The GEMOX combination may be used instead in patients with renal function impairment [76]. Additional combinations of chemotherapeutic agents are being investigated in the first-line setting, including more intensive, triple chemotherapy combinations like GEMCIS with nab-paclitaxel[135]. Acelarin is a NA which can achieve greater intracellular concentrations than gemcitabine and its efficacy in combination with cisplatin is currently being investigated in a phase III study as opposed to GEMCIS (NCT04163900).

After disease progression under first-line chemotherapy with GEMCIS, the next best choice is probably the combination of folinic acid, fluorouracil and oxaliplatin (FOLFOX). In the phase III ABC-06 trial, 162 patients who had already signs of disease progression under GEMCIS administration, were randomly assigned to active symptom control alone or concomitant FOLFOX[136]. In this study, differences in 6- and 12-mo survival (35% *vs* 50.6% and 11.4% *vs* 25.9%, respectively) were considered clinically important, although there was no significant difference in the median OS between the two arms (5.3 mo *vs* 6.2 mo).

A recent multicenter study used clinical and pathological data in addition to information from RNA sequencing in order to identify distinct subtypes of iCCA that might have favorable response to specific treatments. In the subtype that was associated with hepatitis B and C, an *in vivo* sensitivity to gemcitabine was observed. This was attributed to a higher expression of gemcitabine-response genes (SLC28A1 and SLC29A1)[137]. However, these findings should be confirmed in large clinical studies prior to establishing a recommendation for patients with hepatitis B.

Locoregional treatments

Locoregional therapies have implicit appeal as a treatment option for iCCA, as it is a PLC and often the disease burden is either completely or largely confined to the liver [138]. The most widely used types of locoregional therapeutic regimens in iCCA are TACE, radiofrequency ablation (RFA) and microwave ablation (MWA) and transarterial radioembolization (TARE) with Yttrium^{90} (Y-90). The possible role of these modalities in the management of iCCA has mainly been investigated by retrospective studies in various settings. Consequently, the results of the studies are difficult to

interpret, due to the high heterogeneity in terms of patient population (chemotherapy naïve or not) and type of treatment.

TACE is the most widely used treatment modality for patients with intermediate stage HCC not eligible for curative treatments. The rationale for TACE is based on the hypervascular nature of HCC that enables both strong cytotoxic and ischemic effects following the intra-arterial infusion of a cytotoxic agent and the selective embolization of the blood vessels feeding the tumor. However, iCCA rarely appears hypervascular on CT or MRI studies[68]. Despite that, a retrospective study showed that TACE (n = 72) could be beneficial in terms of OS to patients with unresectable iCCA when compared to supportive care (n = 83, OS = 12.2 *vs* 3.3 mo, respectively)[139]. However, in another multiinstitutional retrospective study of 188 patients with advanced-stage iCCA, TACE did not seem to have any significant benefit when compared to other arterial-based therapeutic interventions[138]. The efficacy of TACE has also been investigated in the adjuvant setting by a prospective study of 553 consecutive patients (122 TACE *vs* 431 non-TACE)[140]. Although in the whole cohort, the 5-year OS rates between groups were significantly different (38.4% *vs* 29.7) after 1:1 propensity score matching no significant differences were observed regarding OS or 5-year recurrence rate. Nevertheless, a recent systematic review and meta-analysis concluded that TACE in the adjuvant setting can actually prolong both the OS and the relapse-free survival [141]. A small, prospective study from China included 42 either HBsAg(+) or HBcAb(+) patients with iCCA who had received SR with curative intent and evaluated the prognostic impact of postoperative TACE[118]. TACE was performed in 9 patients who had demonstrated early recurrence of the tumor within 12 mo from the surgery. The OS of the patients in the TACE (n = 9) group was significantly prolonged (1-year, 88.9%; 3-year, 77.8%; 5-year, 66.7%) as compared to the non-TACE (n = 33) group (1-year, 63.6%; 3-year, 30.8%; 5-year, 13%), implying that TACE could have a role in the management of patients with HBV-associated iCCA when performed in the adjuvant setting.

A theoretical advantage of ablative therapies like RFA and MWA when compared to TACE, is that they are safe and effective for the local control of hepatic malignancies in patients considered unsuitable for SR without regard to the tumor's vascular profile [142,143]. The efficacy of ablative therapies in the management of unresectable iCCA has been mostly studied in small-scale, retrospective studies. RFA and MWA seem to be helpful for smaller tumors (< 3 cm), but lose part of their effectiveness when it comes to bigger or irregularly shaped tumors resulting in higher recurrence rates[144, 145]. This issue may be overcome by using stereotactic RFA, which combines pre-interventional three-dimensional imaging, computerized three-dimensional planning of electrode positions, and guided electrode placement at arbitrary angulations and orientations[146,147]. In a systematic review and meta-analysis regarding the efficacy of ablative therapies in the management of unresectable iCCA the pooled 1-, 3-, and 5-year OS rates were 76%, 33% and 16%, respectively[148]. It should be noted though, that this systematic review analyzed studies that included cases with various disease stages, such as recurrent or inoperable disease, thus resulting in a high degree of heterogeneity. To our knowledge, there are no published studies regarding the use of ablative therapies in the context of HBV-associated ICCA. It is obvious that in order to extract safer conclusions regarding the role of ablative therapies in the management of iCCA and HBV-associated iCCA in particular, well-designed prospective studies are needed.

In a systematic review and pooled analysis assessing the efficacy of Y-90 in the management of unresectable iCCA, the median OS was 15.5 mo, but the study highlighted the need for prospective studies on this subject[149]. In the MISPHEC study, a phase II single-arm trial where 41 patients were treated with Y-90 and GEMCIS, the results were rather promising, as median OS was 22 mo and 9 patients (22%) were even downstaged to surgery[150]. A phase III randomized trial investigating the efficacy of Y-90 with GEMCIS compared to GEMCIS alone is currently ongoing (NCT02807181). Unfortunately, there are no published studies regarding the use of TARE in the treatment of HBV-associated iCCA.

Genome-targeted therapies

As our knowledge broadens regarding the DNA alterations and the molecular pathways that are involved in the pathogenesis of HBV-associated iCCA, more possible therapeutic targets are going to arise, until we reach a point where the treatment of each patient will be personalized. As mentioned earlier, some of the most common findings in the genome of viral hepatitis-associated iCCA, include IDH1, IDH2 and FGFR2 mutations. The efficacy of ivosidenib, a targeted inhibitor of IDH1 and IDH2, was investigated in the phase III ClarIDHy trial involving 185 patients with

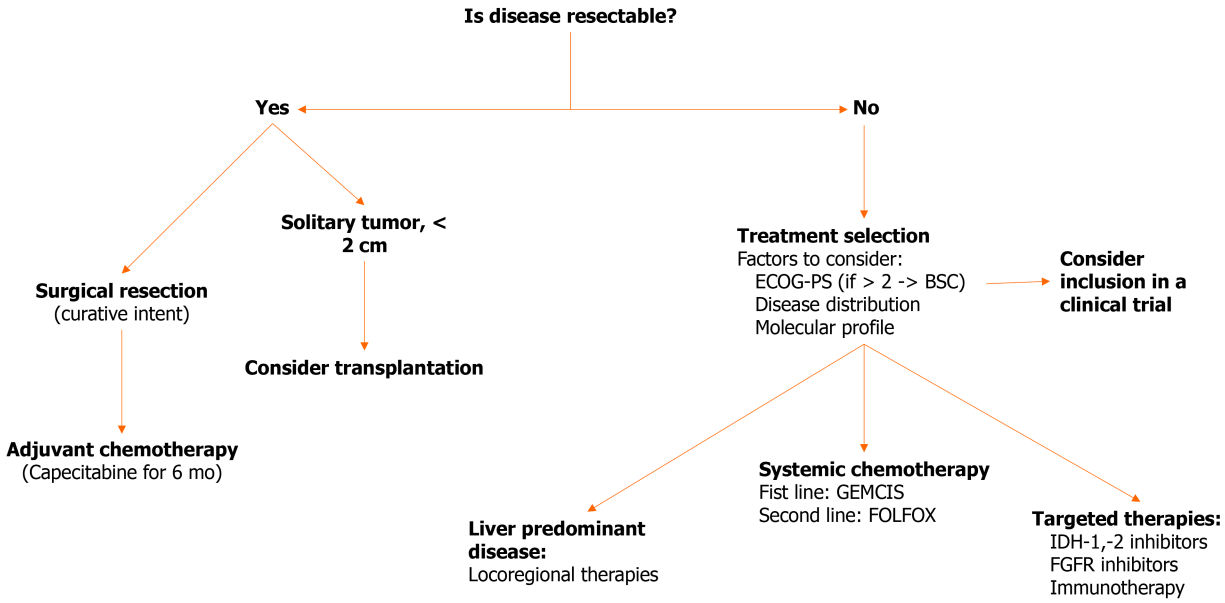


Figure 4 Therapeutic algorithm for hepatitis B virus-associated intrahepatic cholangiocarcinoma. BSC: Best supportive care; ECOG-PS: Eastern Cooperative Oncology Group-Performance Status; FGFR: Fibroblast growth factor receptor; FOLFOX: Folinic acid, Fluorouracil and Oxaliplatin combination; GEMCIS: Gemcitabine and cisplatin combination; IDH: Isocitrate dehydrogenase.

chemotherapy-refractory, IDH1 mutant CCA who were randomly assigned to ivosidenib or placebo[151]. The study managed to prove the clinical benefit of targeting IDH1 mutations in such tumors, as progression-free survival was significantly improved, compared to the placebo arm (HR = 0.37, $P < 0.0001$). Inhibitors of FGFR have been tested mostly in smaller, open-label, phase II trials showing some promising results in the treatment of chemotherapy refractory CCA with FGFR alterations[152-154]. In April 2020, the U.S. Food and Drug Administration approved pemigatinib, an FGFR inhibitor for the treatment of adults with previously treated, unresectable locally advanced or metastatic CCA with FGFR2 fusion or other rearrangement. Some additional FGFR inhibitors are currently being evaluated in the first-line setting, such as pemigatinb in the FIGHT-302 study (NCT03656536) and BGJ398 (oral infigratinib) in the PROOF study (NCT03773302).

Immunotherapy

The clinical data regarding immunotherapy in the management of CCA are limited. Although there is a lot of merit to the idea of a therapeutic approach that targets the immune checkpoint hyperactivation, which is common especially in HBV-associated iCCA, to date, the results from clinical trials are not promising. Pembrolizumab monotherapy provided antitumor activity in just 6% of patients with advanced biliary cancer enrolled in the KEYNOTE-158 study, with median progression-free survival of 2 mo[155]. Possibly, these therapeutic agents can provide better results when administered in combination with chemotherapy. There is an ongoing, phase II, single-arm trial assessing the efficacy of GEMCIS with pembrolizumab in patients with advanced biliary cancer (NCT03260712). Other studies have investigated the possible benefit of immune checkpoint inhibitors combined with locoregional therapeutic regimens (NCT02821754, NCT04299581).

Figure 4 presents a therapeutic algorithm that encompasses all the above described treatment modalities for iCCA.

CONCLUSION

Increasing data during the last 5 years have strengthened the association between the infection with HBV and the development of iCCA. The epidemiological data unveiling this association are from both Eastern and Western countries and this linkage has been confirmed by multiple meta-analyses. In addition, the pathogenetic pathways through which HBV leads to the development of iCCA have been recently the targets of intense investigation. Furthermore, the diagnosis of iCCA in the setting of chronic liver

disease is frequently challenging and often requires histological examination. Nonetheless, certain aspects of this association still remain ill-defined.

In terms of management, the need for effective treatment modalities beyond SR is critical both in the first and the second line treatment. The evolution of personalized approaches through the recognition of specific therapeutic targets will improve the effectiveness of our treatment approaches. It is also likely that the results of ongoing studies in the field of immunotherapy will allow the use of this promising treatment in patients with iCCA, as well. Furthermore, it is currently unclear whether antiviral treatment for HBV can decrease the incidence of iCCA. Large, well-designed studies should address this important question. Focused research on these aspects of the management of HBV-associated iCCA in the future will enhance our ability to manage successfully this dreadful cancer.

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REVIEW

Viral infections in inflammatory bowel disease: Tips and tricks for correct management

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Abstract

Over the past decades, the treatment of inflammatory bowel diseases (IBD) has become more targeted, anticipating the use of immune-modifying therapies at an earlier stage. This top-down approach has been correlated with favorable short and long-term outcomes, but it has also brought with it concerns regarding potential infectious complications. This large IBD population treated with immune-modifying therapies, especially if combined, has an increased risk of severe infections, including opportunistic infections that are sustained by viral, bacterial, parasitic, and fungal agents. Viral infections have emerged as a focal safety concern in patients with IBD, representing a challenge for the clinician: they are often difficult to diagnose and are associated with significant morbidity and mortality. The first step is to improve effective preventive strategies, such as applying vaccination protocols, adopt adequate prophylaxis and educate patients about potential risk factors. Since viral infections in immunosuppressed patients may present atypical signs and symptoms, the challenges for the gastroenterologist are to suspect, recognize and diagnose such complications. Appropriate treatment of common viral infections allows us to minimize their impact on disease outcomes and patients' lives. This practical review supports this standard of care to improve knowledge in this subject area.

Key Words: Inflammatory bowel diseases; Viral infections; Opportunistic infections; Standard of care; Crohn's disease; Ulcerative colitis

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Core Tip: The inflammatory bowel disease population treated with immune-modifying therapies is at increased risk of severe infections, including opportunistic infections sustained by viral agents. Of these opportunistic infections, 40% are due to viral pathogens, including hepatitis A virus, hepatitis C virus, hepatitis B virus, human papillomavirus, influenza virus, human immunodeficiency virus, herpes simplex virus, cytomegalovirus, varicella zoster virus, Epstein-Barr virus, and severe acute respiratory syndrome coronavirus 2 (causing coronavirus disease 19). A challenge for the gastroenterologist is to adopt preventive measures, recognize and treat common viral infections to minimize their impact on disease outcomes and patients' lives. This practical review supports this standard of care, aiming to bridge the gap of knowledge in this subject area.

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INTRODUCTION

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC) are lifetime conditions characterized by a relapsing and remitting clinical course. The complete pathogenic mechanisms of IBD are unknown. The major hypothesis is that the diseases occur when genetically vulnerable individuals meet unknown environmental triggers that exacerbate an inappropriate immune response against gut microbiota[1]. Curative therapies for IBD are not available yet. Medical treatment aims to guarantee long-lasting disease remission, thus avoiding complications and improving patient quality of life.

Drugs currently available for the management of IBD are mesalazine (5-ASA), locally active steroids, systemic steroids, thiopurines such as azathioprine (AZA) and mercaptopurine (MP), methotrexate (MTX), and biological therapies [Tumor necrosis factor-alpha inhibitors (anti-TNF) and other monoclonal antibodies targeting interleukin 12 (IL-12), IL-23, and cellular adhesion molecule ligands a4 integrin and a4b7 integrin][2].

Over the past decades, IBD treatment has become more targeted, anticipating the use of immune-modifying therapies at an earlier stage[3,4]. This has been correlated with favorable prognosis such as a reduction in surgery, hospitalization, and use of steroids[5-10], but it has also brought with it concerns about potential infectious complications.

IBD patients treated with immune-modifying therapies have an increased risk of developing severe infections, including opportunistic infections sustained by viral, bacterial, parasitic, and fungal agents[11-16], that are associated with hospital admission, use of intravenous antimicrobials, disability and death. The incidence of serious infections is not well defined, ranging from 10 to 100 events per 1000 patient-years[13,17-19]. Opportunistic infections are caused by ordinarily nonpathogenic organisms that can take advantage of an impaired immune system. According to the European Crohn's and Colitis Organization (ECCO) consensus guidelines on the prevention, diagnosis and management of opportunistic infections in IBD[20], updated in 2014[12], IBD patients treated with immune-modifying agents, especially in combination, those with malnutrition, comorbidities and a history of severe infections should be considered at risk for opportunistic infections. In an era of intensifying immune-modifying therapies, infective complications have emerged as a focal safety concern in patients with IBD. Today's challenge for gastroenterologists is to adopt preventative strategies, suspect, recognize, and treat appropriately IBD infectious complications to minimize their impact on disease outcomes and patients' lives. In particular, viral infections represent demanding problems for the clinician, as they are often difficult to diagnose and are associated with significant morbidity. This review intends to outline the most relevant viral infections in IBD, focusing on the careful

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screening and monitoring before and during the use of immune-modifying therapies, timing of vaccinations, opportunity for primary or secondary prophylaxis and indications for therapy. A summary of recommendations for the prevention and management of viral infections in IBD are shown in Table 1.

VIRAL INFECTION IN IBD

Risk factors

A population-based study of patients with IBD[18] found that of opportunistic infections, 40% were due to viral pathogens. A recent prospective observational study [21] estimated that the incidence rate of systemic viral infections in patients with IBD was 2 per 1000 person-years, three-fold higher compared to the general population. Age is an independent risk factor for opportunistic infections in IBD[22], but for viral infections, the highest incidence rates are observed in patients under the age of 35 years[21]. In elderly patients, infections sustained by viral pathogens are rare compared with the younger population[23], except for influenza[24], herpes zoster reactivation (shingles)[25], and viral gastroenteritis[26]. Viral infections may also be triggered by disease activity and the resulting defective mucosal immunity, especially cytomegalovirus (CMV) colitis[27,28], and Epstein-Barr virus (EBV) systemic reactivation[29]. Finally, viral infections may arise as adverse events attributable to the immune-modifier action of IBD drugs. The direct correlation between a specific immunomodulator or biologic drug and viral infections has not been established. A retrospective analysis found that AZA/6MP-treated patients are at risk of developing opportunistic viral infections, such as herpes simplex (HSV), varicella-zoster (VZV), CMV, and EBV[11]. In a prospective cohort of outpatients with IBD, it was reported that exposure to thiopurines was associated with an increased incidence of cutaneous herpes flares and warts[30]. The incidence of zoster is increased in patients with IBD [31-33] and exposure to immunomodulators, in particular corticosteroids or combination therapy with thiopurines and anti-TNF agents, raises the risk[18,34]. During a 5-year follow-up, combination therapy (anti-TNF + thiopurine) was associated with an increased risk of opportunistic viral infections compared to anti-TNF monotherapy (1.3% *vs* 0.7%); no difference was found compared to thiopurine monotherapy (1.1%). Conversely, anti-TNF monotherapy was associated with a decreased risk of opportunistic viral infection compared to thiopurine monotherapy, suggesting that thiopurines drive the risk of opportunistic viral infections under combination therapy. These observations seem to agree with the AZA/6MP mechanism of action which primarily suppresses T lymphocytes activity that is involved in the prevention of viral infections.

With regard to corticosteroids, it is difficult to conclude their benefit and risk as they have many selection biases, as they are likely to be given to patients with severe disease, and there is heterogeneity in the type, dose and duration of their use. There are data suggesting that doses of prednisolone above 20 mg are associated with increased risk of bacterial and viral infections in IBD[35]. In patients with IBD exposed to thiopurines and anti-TNF agents, the increase in mortality from infection is negligible[17,36], instead, some studies have reported increased mortality in patients exposed to corticosteroids[34,37]. Case series recorded thiopurine-associated fatal infections, mainly in young patients. These infections include severe forms of varicella [38] and primary EBV or CMV infections complicated by hemophagocytic lymphohistiocytosis[39,40].

General approach

In immunocompromised patients, fever is sometimes the only manifestation of severe infection[41]. The approach in febrile patients must include exploration of their history and accurate physical examination. It is essential to recognize any symptoms or signs that can help identify the site of infection. Evaluation tests should include complete blood cell counts, C-reactive protein, serum procalcitonin (PCT), urine analysis and culture, VZV serology in patients without a reliable history of varicella immunization, hepatitis B virus (HBV) and hepatitis C virus (HCV), EBV and human immunodeficiency virus (HIV) serologies, stool examinations and strongyloidiasis serology (for returning travelers) and chest X-ray[12]. In the case of severe illness or when unusual pathogens are suspected, an infectious disease specialist should be involved.

In patients with respiratory symptoms (dyspnea, cough, purulent sputum, hemoptysis, pleuritic chest pain) or focal chest signs, a chest X-ray or chest ultrasound should be performed, and oxygen saturation determined. For patients with abnormal

Table 1 Summary of recommendations for the prevention and management of viral infections in inflammatory bowel disease

Infection	Screening prior to IM	Vaccination	Prophylaxis	Diagnosis	Therapy
HAV	IgG anti-HAV	Inactivated HAV vaccine; (2 doses, 0-6 mo)	-	IgG anti-HAV	Supportive
HCV	Ab anti-HCV; If positive HCV-RNA	-	-	Anti-HCV Ab; if positive HCV-RNA	DAA[62]
HBV	HBsAg, anti-HBs, anti-HBc;If positive HBV-DNA	Accelerated double-dose; (0, 1, 2 mo); If no response, re-vaccination; (0, 1, 2 mo) at a double-dose	In HBsAg+ (or antiHBc+); Entecavir 0.5 mg/d;Tenofovir, start 2 wk prior to IM	Exacerbation: ↑ AST/ALT; 100-fold rise HBV DNA	Entecavir 0.5-1 mg/daily; Tenofovir
HPV	Cervical smear test	bi/quadri/nine-valent;Women: 9-26 yr, Men: 11-23 yr	-	Cervical smear test	-
Influenza	-	Inactivated non-live trivalent	-	RT-PCR	Single neuraminidase inhibitor
HIV	HIV p24 Ag and Ab	-	-	Acute infection: RT-PCR	ART[99]
HSV	History of herpes lesions	-	Frequent/severe recurrence:acy-, valacy-, famci-clovir	Viral culture, H&E, RT-PCR	acyclovir, valacyclovir, and famciclovir
CMV	In steroid refractory patients	-	-	CMV inclusions in H&E + IHC followed by tissue RT- PCR	IV ganciclovir 5-7.5 mg/kg twice daily for 2 wk
VZV	VZV IgG/IgM	VZV vaccine: 4-3 wk before IM; HZ vaccine (recombinant): 2 doses, 0-3/6 mo	After exposure: VZV-Ig	RT-PCR on skin lesions	IV or PO acyclovir, valacyclovir, and famciclovir
EBV	EBV IgG/IgM	-	-	IgM VCA + and IgG EBNA -	-
SARS-CoV2	Recommended (test based on availability)	Recommended; mRNA-based; adenoviral vector	-	nasopharyngeal swabs; PCR-SARS-CoV-2	-

IM: Immune-modifier; DAA: Direct-acting antiviral; RT-PCR: Real-time polymerase chain reaction; IV: Intra-venous; PO: Per-os; HAV: Hepatitis A virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HPV: Human papillomavirus; HIV: Human immunodeficiency virus; HSV: Herpes simplex virus; CMV: Cytomegalovirus; VZV: Varicella zoster virus; EBV: Epstein-Barr virus; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

oxygen saturation rates, computed tomography is required[42]. When a radiological and clinical picture of pneumonia is detected, an etiological diagnosis may be achieved by performing hemocultures, sputum cultures, nasopharyngeal swabbing for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) or influenza virus real-time polymerase chain reaction (RT-PCR), Legionella and pneumococcal urinary antigen or, in specific situations by bronchoalveolar lavage.

The persistence or relapses of gastrointestinal symptoms (diarrhea, rectal bleeding, abdominal pain and weight loss) may be due to exacerbation of the underlying IBD or enteric infections. As a first approach, a potential enteric infection should be ruled out. In this regard, stool cultures with examination for parasites and *Clostridium difficile* toxin testing are necessary[43]. Recently, different multiplex molecular assays (FilmArray Gastrointestinal GI Panel and Luminex xTAG Gastrointestinal Pathogen Panel, GPP) have been developed for the rapid identification of pathogens responsible for causing diarrheal illness, included Adenovirus F 40/41, Astrovirus, Norovirus GI/GII, Rotavirus A, and Sapovirus (I,II, IV, and V). Their adoption in clinical practice can improve the diagnostic efficiency of GI pathogens[44]. If stool microbiological analyses are negative, colonoscopy or recto-sigmoidoscopy with biopsies should be performed. HSV, and especially CMV, may be responsible for severe IBD relapses.

A lumbar puncture should be considered, under neurological advice, in patients with meningeal signs or encephalopathy, with strain culture or serology of cerebro-spinal fluid (CSF). CSF cultures are essential for the detection of bacterial pathogens. PCR of CSF is the gold standard for CMV, HSV, VZV, *Toxoplasma gondii* and John Cunningham virus (JCV) diagnosis. Magnetic resonance imaging should be performed for patients with mass forming lesions or encephalopathy and may be effective for diagnosing progressive multifocal leukoencephalopathy due to JCV, reported in the past for patients treated with natalizumab[45].

Dermatological manifestations of viral infections are mainly caused by HSV and VZV. The diagnosis of these conditions is usually based on clinical manifestations. Yet virological analysis can be performed directly on a recent lesion by PCR.

Diagnostic approaches for IBD patients with infectious symptoms are shown in Table 2.

IBD patients should follow all age-appropriate vaccinations as recommended by the Advisory Committee on Immunization Practices[46]. It is also important to respect the optimal timing of vaccination with regard to initiation of immunosuppressive therapy, as the use of immunosuppressive agents can lead to decreased immunogenicity of the vaccination[47,48].

In the case of minor infection (rhinitis, upper respiratory tract infection, *etc*.) with no risk of disseminated disease or rapid worsening, it is unnecessary to withdraw immunomodulators. However, in the case of risk for disseminated or uncontrolled disease (shingles, viral pneumonia, encephalitis), high-grade fever and infection with a well-known mortality risk, withdrawal of immunomodulators are best advised at the peak of infection, and the decision to resume immunomodulator treatment is on a case-by-case basis and a multidisciplinary approach[49].

VIRAL DISEASES

Hepatitis A virus

Hepatitis A virus (HAV) is responsible for hepatitis A infection, which is usually a mild, self-limited disease that does not become chronic. It is transmitted by the fecal-oral route, and poor hygienic and socioeconomic conditions promote this infection[50,51]. Infection confers lifelong immunity and is preventable *via* vaccination. The onset of symptoms is sudden, with malaise, asthenia, nausea, vomiting, and pain in the upper abdominal quadrants. No specific disease manifestations in immunocompromised hosts have been described. The most relevant biochemical alteration is the increase in transaminases (often > 1000 IU/dL) and, generally, precedes the increase in bilirubin (typically ≤ 10 mg/dL). The presence of IgM anti-HAV antibodies allows the etiological diagnosis. The American College of Gastroenterology and the Korean Association for the Study of Intestinal Diseases recommend a test for HAV (IgG anti-HAV antibodies) in patients with IBD. In those who are negative, vaccination should be recommended[52,53]. On the other hand, ECCO guidelines suggest vaccination only in high-risk subjects and those traveling to endemic areas[12]. Inactivated hepatitis A vaccines should be preferred[54], as they have high, long-lasting immunogenicity with few side effects[55]. Since immunosuppressive therapy can lower the seroconversion rate[56], optimal timing for HAV vaccination is at IBD diagnosis or before starting immunosuppressive therapy, even if administration is acceptable during maintenance therapy. The vaccine should be administered in 2 doses with an interval of 6 mo.

HCV

The global HCV prevalence is estimated at 2.5%, ranging from 2.9% in Africa and 1.3% in the Americas[57]. In Europe it is estimated that 0.2-2% of the population is infected with HCV. The prevalence of HCV in patients with IBD is comparable to that in the general population[58-61]. HCV is typically transmitted parenterally. Acute HCV infection is often asymptomatic and chronic HCV infection develops in about 85% of all cases. Among patients with chronic HCV infection, 20% develop liver cirrhosis within 20 years of disease duration, with a high rate of incidence of hepatocellular carcinoma. General measures to reduce or prevent HCV infection are appropriate since vaccination and prophylactic treatment are not available. The ECCO guidelines suggest performing HCV screening before starting treatment with immune-modifying drugs for IBD. Testing should be performed by searching for anti-HCV antibodies and, if antibodies are positive, by identification of HCV-RNA[62,63]. Management of HCV in this population is important as immunosuppression may precipitate HCV-associated liver damage[64] and immunomodulators may result in cumulative liver toxicity[65,66]. If infection is confirmed, patients should be treated according to the HCV clinical practice guidelines[64], possibly before starting biologics or immunomodulator therapy. However, the timing strategy for treating HCV-infected IBD subjects could depend on several factors, including the IBD activity and patient's comorbidities. If HCV-infected patients with IBD cannot delay their immune-modifying therapy, their liver function should be monitored closely. There are no data to suggest that biologics are associated with reactivation or exacerbation of the course

Table 2 Diagnostic approach to inflammatory bowel disease patients with infectious symptoms

Symptoms cluster	Potential viral pathogens	Diagnostic work up
Fever	VZV, HBV, HCV, EBV, HIV, SARS-CoV-2	Medical history, physical examination
		Blood cell counts, PCR, PCT
		VZV, HBV, HCV, EBV and HIV Ab
		Hemocultures
		Urine analysis and culture
		Stool examinations
		Strongyloidiasis serology
		Chest X-ray
		Infectious disease specialist consult
Respiratory	Influenza, SARS-CoV-2 (Bilateral interstitial infiltrates)	Chest X-ray or US
		SaO_2% < range → CT
		Pneumonia:
		Sputum cultures
		NPS for SARS-CoV-2/influenza virus Legionella and pneumococcal U-Ag
		Bronchoalveolar lavage
Gastrointestinal	HSV, CMV, EBV, Adenovirus, Astrovirus, Norovirus, Rotavirus, Sapovirus	Rule out potential enteric infection:
		Stool cultures
		Examination for parasites
		C. difficile toxin testing
		Multiplex molecular assays
Central nervous system	CMV, HSV, VZV, JC virus	Neurological advice
		CSF cultures and PCR
		MRI
Cutaneous	HSV, VZV	Dermatological consult
		PCR on recent lesion

CRP: C-reactive protein; PCT: Procalcitonin; US: Ultrasound; CT: Computed tomography; NPS: Nasopharyngeal swab; U-Ag: Urinary antigen; CSF: Cerebrospinal fluid; PCR: Polymerase chain reaction; MRI: Magnetic resonance imaging; HAV: Hepatitis A virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HPV: Human papillomavirus; HIV: Human immunodeficiency virus; HSV: Herpes simplex virus; CMV: Cytomegalovirus; VZV: Varicella zoster virus; EBV: Epstein-Barr virus; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; JC: John Cunningham virus; *C. difficile*: *Clostridium difficile*.

of HCV and the safety profile of anti-TNF-α agents in HCV patients is good, even if there seems to be variances in the hepatotoxic profile between different biologics[59,66,67].

HBV

HBV is carried globally by 248 million people. HBsAg seroprevalence is 3.61% and it varies by country and region, from 2% in areas with low prevalence (UK, Canada, Western Europe, *etc.*), 2%-7% in those with moderate prevalence (South Korea, Mediterranean countries, Japan, Central Asia, Middle East, and parts of South America), and more than 8% in countries of the African region, which have the highest endemicity[68,69]. The prevalence of HBV in patients with IBD is similar to that in the general population[61,70]. HBV can manifest as acute and chronic infection. In the acute phase, the infection can manifest as jaundiced or non-jaundiced hepatitis (high levels of HBV DNA are associated with increased transaminases, with varying degrees of bilirubin elevation) and in more severe cases as fulminant hepatitis. The chronic phase has various patterns of expression, from asymptomatic carrier to the sequence chronic hepatitis, liver cirrhosis and related hepatocellular carcinoma. Patients with

IBD should be assessed for HBV infection (HBsAg, anti-HBs, anti-HBc) or immunization status. Anti-core antibodies could represent the only indicator in patients with HIV or HCV co-infections[71]. In patients diagnosed with HBV infection, HBeAg, anti-HBe, and HBV DNA should also be assessed[12] and then referred to a specialist for diagnosis of the phase of HBV infection. Vaccination of seronegative (anti-HBs -, anti-HBc -) patients is recommended. Patients with IBD on immunosuppression demonstrated a significantly reduced response to the standard vaccination (rHBAg 20 μg single dose at 0, 1 and 6 mo) as compared with the general population[72] (64% adequate immune response and 40% effective immune response)[73]. Receiving an accelerated double-dose at 0, 1, 2 mo followed by revaccination (0, 1, and 2 mo) at a double-dose if no adequate response is achieved has demonstrated better efficacy than the standard schedule[74,75]. Serology testing 1 to 2 mo after administration of the last dose are required to assess the need for revaccination and check-ups for anti-HBs yearly or every 2 years seems to be good practice[12,76]. A single booster dose should be given to immunocompromised patients who lose seroprotection[77].

The risk of viral reactivation in HBV is increased in patients who are receiving immune-modifying therapy and are HBsAg-positive or HBsAg-negative plus anti-HBc positive[64], in a manner that is proportional to the level of immunosuppression achieved[65,78]. Liver dysfunction has been described in retrospective studies in 25%-36% of HBsAg-positive patients with IBD receiving immunosuppressive agents; HBV reactivations have been associated with hepatic decompensation in a considerable proportion of cases[79,80]. Recognized risk factors were treatment with 2 or more immunomodulators for a long period of time, presence of HBV DNA and avoidance of antiviral prophylaxis. TNF-α and related cytokines are important in regulating hepatitis B replication and anti-TNF treatments confer a high risk of HBV reactivation [81,82].

Exacerbation of HBV infection is recognized by an increase in transaminases associated with high levels of serum HBV DNA (100-fold rise compared to baseline) in patients with chronic hepatitis B. HBsAg positive patients should receive prophylactic antiviral treatment with nucleotide/nucleoside analogues with a high barrier to resistance (*i.e.* entecavir or tenofovir), best started 2 wk prior to the introduction of immunomodulators or a biologic and continued for 12 mo after their withdrawal.

Relapse of past HBV infection is detected based on reverse seroreversion from HBsAg-negativity to HBsAg-positivity, or on the detection of serum HBV DNA in patients who are HBsAg-negative plus anti-HBc-positive. Relapse of occult HBV has been described in IBD and rheumatology patients treated with anti-TNF and corticosteroids or DMARDs[83,84], but this rarely occurs[59,79]. The preventive approach in patients with HBsAg negative and anti-HBc positive varies among the published guidelines. The American Gastroenterological Association suggest antiviral prophylaxis for patients treated with anti-TNF-α or with corticosteroids[85]. The ECCO, according to the European Association for the Study of the Liver, recommends active monitoring of AST/ALT levels and the virus markers (HBsAg and/or HBV DNA) every 1-3 mo with antiviral therapy once HBV DNA or seroreversion is detected (preemptive therapy strategy)[12,64].

Human papillomavirus

Human papillomavirus (HPV) is a sexually transmitted infection. There are approximately 40 serotypes of HPV classified into low-risk serotypes and high-risk serotypes, which are associated with cervical and anal squamous cell carcinoma[86]. HPV also causes cutaneous warts. There are reports of an increased frequency of HPV-related anogenital warts in patients receiving AZA[30], but not anti-TNF[87]. IBD has not been associated with the development of cervical cancer; however, patients with CD who smoke, are younger at diagnosis, and those who use thiopurines or methotrexate combined with corticosteroids might be more at risk[88,89]. Other immunosuppressive medications, in particular anti-TNF therapy, seem not to have an increased risk. However, the quality of evidence is poor, physician awareness and prevention by lifestyle counseling, HPV vaccination and screening are warranted[90]. There are three types of HPV vaccine: bivalent, Cervarix, comprising 16, 18 serotypes, quadrivalent, Silagard, and Gardasil, with 6, 11, 16, 18 serotypes, and nine-valent, Gardasil 9, with 6, 11, 16, 18, 31, 33, 45, 52, 58 serotypes. As the HPV vaccines do not contain live viruses, they may be administered to an immunosuppressed patient with an excellent safety profile and immunogenicity[91]. The vaccine is indicated for women aged 9 to 26 years, preferably before the first sexual intercourse but also after initiation of sexual activity. Male patients should be vaccinated at the age of 11 to 12 years, and catch-up for those aged 13 to 21 years. Of note, cervical screening recommendations are similar for vaccinated and nonvaccinated women. In IBD patients, a cervical smear test should

be performed initially at the time of diagnosis, and if normal then annually[12]. Different studies have shown an unsatisfactory cervical cancer screening rate and inadequate HPV vaccine coverage among IBD patients[92-94] advising that better cooperation between patients and physicians is required to improve education on preventive measures. Management of abnormal findings at cervical smears includes colposcopic examination with biopsy/brushing, and eventually conization.

Influenza virus

There are two types of influenza virus: type A and type B, which cause seasonal epidemics of acute respiratory illness. IBD patients have an increased risk of influenza compared with those without IBD with a higher rate of hospitalization, often with bacterial pneumonia superinfection[95,96]. Patients requiring hospitalization for possible influenza infection should be tested. The gold standard for diagnosis of influenza is RT-PCR testing in specimens from different respiratory sites[97]. In the outpatient setting, diagnosis is made with a high likelihood by clinical criteria alone. Inactivated non-live trivalent influenza vaccination is recommended annually in the fall and spring for IBD patients and their household contacts to prevent influenza virus infection. Live attenuated influenza vaccine is not recommended for patients on immunomodulators[12]. IBD patients treated with anti-TNF alone or combined with thiopurine, may have a reduction in the influenza seroprotection rate after inactivated non-live trivalent influenza vaccination[98-100]; however, it is sufficient to warrant annual influenza vaccination. Antiviral treatment (single neuraminidase inhibitor; either oral oseltamivir, inhaled zanamivir, or intravenous peramivir) should be started as soon as possible for patients with documented or suspected influenza, who are taking immunomodulator therapy, especially if older than 65 years[97].

HIV

HIV belongs to the human retrovirus family. HIV infection causes a wide range of clinical consequences varying from asymptomatic to severe opportunistic diseases due to immunosuppression, including infections and malignancies, which are characterized by acquired immunodeficiency syndrome (AIDS). HIV infection is usually transmitted through sexual intercourse, exposure to infected blood, or perinatal transmission. Antiretroviral therapy (ART) effectively suppresses viral replication so that an almost normal immune status can be regained, leading to dramatic reductions in morbidity and mortality. For most individuals, an ART regimen consists of a dual nucleoside combination plus a third agent from a different class[101]. Patients with IBD and HIV infection have shown controversial data concerning the remission hypothesis of IBD due to CD4 count depletion caused by HIV[102]. A recent multicenter retrospective cohort study suggested that HIV infection might attenuate the IBD course as HIV-infected patients need fewer immunosuppressants and biologics to control the disease[103]. In a previous analysis, over a median follow-up of 8.4 years, HIV status was the only risk factor independently associated with a lower probability of IBD relapse[104]. However, evidence of the interrelation between HIV and IBD remains poorly understood. The diagnosis of IBD in HIV infection can be complex because of possible symptom overlap[105]. The differential diagnosis includes a multitude of etiologies from protozoic, fungal, viral, and bacterial pathogens (HSV, AIDS-related CMV gastrointestinal disease[106-108], Salmonella, Shigella, Campylobacter, *Mycobacterium tuberculosis*, Histoplasma or Cryptococcus) to malignancy (lymphoma or Kaposi's sarcoma[109]) to medications (ART-associated diarrhea[110,111]). The entity known as AIDS enteropathy can be diagnosed once other causes of diarrhea are investigated and excluded[112]. It is expected that all cases of IBD in HIV infection require biopsy confirmation by an expert gastrointestinal pathologist. All IBD patients undergoing immunomodulator or biological therapy should receive testing for HIV infection (HIV p24 antigen and antibody testing, with PCR if acute infection is suspected) to exclude unknown infection. Experiences in treating individuals with HIV infection with anti-TNF therapies are limited. It is possible to prescribe with a reasonable ratio of benefits to risks if the patients have a low HIV viral load and are not severely immunosuppressed (>350 CD4 cells/μL), keeping them closely monitored[103,113-115].

HSV

HSV type 1 (HSV-1) and HSV type 2 (HSV-2) are common infections worldwide. HSV-1 is implicated in most cases of orofacial herpes lesions ("cold sores"), while HSV-2 causes most cases of recurrent genital herpes[116]. After primary infection, HSV establishes chronic infection in neural ganglia and reactivates on mucosa and skin;

infection is lifelong. Especially in immunocompromised hosts, HSV infection may cause severe manifestations, including encephalitis, meningitis[117], hepatitis[118], respiratory tract infections[119,120], esophagitis[121-123] and proctitis[124-126]. Within the treatment regimens for IBD, corticosteroids and azathioprine have been related to skin or genital herpetic flares[30,127]. Extended HSV colitis associated with IBD exacerbations are largely reported[128-132]. The testing approach depends upon the site of the disease (mucocutaneous, ocular, neurologic or visceral). HSV can be diagnosed, either by isolating the virus in a culture of the affected tissue or by histopathological examination showing nucleated ground-glass herpes inclusions in the cells. HSV PCR is an alternative, but more sensitive method to confirm HSV infection. Before starting with immunosuppressive drugs, previous history of HSV infection should be investigated, but serological screening is unnecessary[12]. Frequent and severe recurrence of HSV disease can be prevented by therapy with oral acyclovir (400-800 mg twice or thrice daily), valacyclovir (500 mg daily) or famciclovir (500 mg twice daily).

CMV

With a worldwide prevalence of over 80%[133], CMV causes in most subjects asymptomatic or mononucleosis-like syndrome during primary infections, persisting lifelong in a latent form in different organs, including the gut[134]. Especially in immunocompromised patients, CMV can reactivate, causing tissue-invasive end-organ damage, mainly in the brain, lung, retina and digestive tract. CMV colitis in IBD patients should be considered. Seroprevalence (CMV IgG) in IBD patients is comparable to that in the general population[135,136]. On the contrary, identification of CMV viral DNA in the mucosa was reported, from limited data, to be more prevalent in patients with IBD than in healthy controls[137,138]. CMV tissue reactivation is rare in CD flares, whereas it is frequent in UC[139]. Characteristics that increase the risk of CMV reactivations in IBD and develop colitis flare include female sex, pancolitis[140,141], advanced age[142], and disease duration less than 60 mo. Immunomodulator therapy with azathioprine[140,143] and steroids[142,144] is a recognized trigger factor, while on chronic anti-TNF, the data is conflicting[145-148]. Screening for subclinical CMV infection in IBD patients is not required unless the patient is steroid-refractory[12]. Patients with CMV infection and severe acute colitis, in fact, show a higher probability of steroid resistance[135,136,149] and enhanced risk for colectomy[150,151] than non-infected patients. Colonic CMV infection can be diagnosed by hematoxylin and eosin (H&E) or immunohistochemical staining (IHC) histology, serology assay, polymerase chain reaction (PCR) for CMV DNA in peripheral blood or tissue (from targeted biopsies taken from the ulcer base), and CMV antigenemia (pp65)[152]. The gold standard for diagnosing CMV overinfection is the identification of CMV inclusions (typically basophilic intranuclear inclusions) or positive CMV-specific immunohistochemistry staining on histopathology[153], eventually followed by tissue-direct PCR for viral load quantification. CMV infection cannot be excluded based on a negative whole-blood PCR result[154]. In patients who are steroid-dependent or refractory[155] and those with high CMV viral load in colonic tissue[156], antiviral therapy is recommended. Several agents are available for CMV infection, including ganciclovir, valganciclovir and foscarnet. The most used antiviral agent is intravenous ganciclovir at a dose of 5-7.5 mg/kg twice daily for 2 wk [152]. ECCO guidelines allow a course of intravenous ganciclovir 5 mg/kg twice for 3-5 days, followed by oral valganciclovir at 900 mg *per os* twice daily for 2-3 wk[12]. Foscarnet (for 2-3 wk) is an alternative in case of resistance or intolerance (hematologic or renal toxicity). Overall, there is a lack of knowledge to give recommendations on managing immunosuppressant medications during or after treatment of CMV colitis. Inducing remission with anti-TNF and tapering off the steroids while continuing intravenous ganciclovir may be an acceptable strategy[152].

VZV

VZV causes varicella (chickenpox) during its primary infection, and after decades of latency in sensory ganglia, it maintains the potential for reactivation as herpes zoster (shingles). Primary infection is generally a mild-moderate disease in most children. However, it can be life-threatening in adults (especially during pregnancy) and immunocompromised patients, potentially leading to severe complications such as central nervous system involvement, pneumonia, secondary bacterial infections, and death[157]. At diagnosis, all IBD patients (unless those with documented vaccination history) should be tested for VZV IgG serology[158]. Corticosteroids, thiopurines, methotrexate, and anti-TNF in monotherapy or even more in combination, are recognized risk factors for varicella infection[159,160]. The literature describes 23 cases

of varicella in IBD patients receiving immunosuppressants and the fatality rate is reported to be 22%[161]. Diagnosis is based on clinical signs (fever and typical vesicular lesions in different stages of development on the face, trunk, and extremities), or real-time PCR on samples taken from skin lesions in an immunocompromised host or in the evaluation of atypical lesions[162]. In immunocompromised hosts with active varicella lesions, antiviral therapy is recommended in order to reduce the severity of symptoms and the risk of serious complications. Oral valacyclovir (1 g 3 times daily) may be considered in patients with mild disease followed closely. For most patients, initial therapy with intravenous acyclovir (10 mg/kg every 8 h) is suggested, with the possibility of switching to oral antiviral after defervescence in the absence of visceral involvement. The treatment duration is 7-10 days, and it has to be continued until all lesions have crusted. After significant exposure, primary prophylaxis with VZV immune globulin within 10 days is indicated in IBD patients receiving immunosuppressive therapy since they are ineligible for varicella vaccine prophylaxis[163]. From a prevention perspective, as the VZV vaccine is a live vaccine, its administration (2 doses, 1 mo apart) should be carried out at least 4-3 wk before starting immunosuppressive therapy[12,52] or 3-6 mo after stopping it.

IBD patients are also at increased risk of developing herpes zoster (HZ). Due to diffuse concurrent use of immunosuppressant agents, clinical manifestations are often more severe, and the risk of complications such as post-herpetic neuralgia, central nervous system and ocular involvement are enhanced[164,165]. Corticosteroids and thiopurines (especially in combination with anti-TNF medication) are known risk factors linked to HZ infection[32,33,166]. Recently tofacitinib has also been observed to be related in a dose-dependent manner to HZ, with additional risk conferred by older age, diabetes mellitus, corticosteroids therapy and prior anti-TNF failure[167,168]. The diagnosis of HZ is usually based on the clinical presentation (unilateral, usually painful vesicular-papular eruption with a defined dermatomal distribution). However, since immunocompromised subjects may have an atypical presentation (hemorrhagic skin lesions affecting multiple dermatomes), real-time PCR may confirm the diagnosis. Antiviral therapy must be started in IBD patients with HZ, even after 72 h from presentation. Patients with disseminated HZ should be hospitalized for intravenous acyclovir therapy (10 mg/kg every 8 h). On the other hand, oral therapy is usually sufficient for the initial treatment of uncomplicated HZ. Regimen options last 7 days and include valacyclovir (1 g 3 times daily), famciclovir (500 mg 3 times daily) and acyclovir (800 mg 5 times daily). Two types of zoster vaccine are available: the first one is a live attenuated vaccine, which is contraindicated in patients receiving moderate or high-dose immunosuppressive therapy and recently a new recombinant glycoprotein E vaccine has become available. The vaccine is administered twice, at 0 and 2-6 mo. Recombinant vaccine has been proven to be highly effective in reducing HZ risk and postherpetic neuralgia among adult subjects[169]; thus, the ACIP recommends it for use in immunocompetent adults aged ≥ 50 years[170]. There are insufficient data to make recommendations regarding zoster vaccination in immunosuppressed patients, but initial evidence suggests good safety and immunogenic profile in this subgroup of patients[171-173]. Therefore, it seems to be an acceptable strategy, to propose recombinant vaccine in patients with uncontrolled severe disease activity, history of shingles, a scheduled therapy with tofacitinib, treated with azathioprine and aged > 40 years, and to all patients aged > 50 years[161].

EBV

EBV is a ubiquitous herpesvirus with a seroprevalence >90% worldwide. It is responsible for infectious mononucleosis (IM), and after primary infection persists asymptomatically lifelong in latently-infected circulating B-lymphocytes[174]. EBV infection is associated with different types of malignancies, including B cell lymphomas, T cell lymphomas, Hodgkin lymphoma and nasopharyngeal carcinomas [175-177]. The incidence rate of lymphoproliferative disorders is much higher in IBD patients than in the general population. The relationship between immunosuppressive drugs (in particular, evidence seems to suggest that thiopurines have specific additional risks[178,179]) and EBV infection may be implicated in the pathogenesis of this disease[180,181]. In addition, severe EBV diseases such as fatal infectious mononucleosis and hemophagocytic lymphohistiocytosis occur when primary infection develops in immunosuppressed patients[182-184]. Therefore, it seems relevant to determine EBV serology status by testing EBV IgG before starting immunomodulator therapy. In EBV seronegative patients, anti-TNF monotherapy should be preferred over thiopurines[12]. The diagnosis of acute EBV infection is made in the presence of IgM VCA and the absence of IgG EBNA antibodies. When primary infection is recognized, immunomodulator therapy should be reduced or discontinued

if possible. Advice from a hematologist/oncologist is required when lymphoproliferative disease is suspected.

SARS-CoV-2 (coronavirus disease 19)

On March 11th, 2020 the World Health Organization declared coronavirus disease 19 (COVID-19) a global pandemic, marking one of the most intense passages in recent history. The range of symptomatic infection goes from mild to critical; >80% of cases are not severe[185], but morbidity and mortality are not negligible. Current evidence shows that the IBD population does not have an increased prevalence of SARS-CoV-2 infection[186-188] and immunomodulators, biologics, or Janus Kinase inhibitors do not represent a risk factor for SARS-CoV-2 infection and more severe COVID- 19[189,190]. On the other hand, in several studies of IBD patients with COVID-19, a trend towards an adverse outcome with concomitant corticosteroids was reported[191]. COVID-19 may occur early with gastrointestinal symptoms (anorexia, nausea, vomiting, abdominal pain, and diarrhea)[192] simulating IBD manifestations. For that reason, patients with a suspected IBD flare should be tested to exclude SARS-CoV-2 infection, especially before initiation of biologics[193]. When IBD patients are diagnosed with COVID-19, in its current state, several consensuses and expert opinions propose discontinuation of thiopurines, methotrexate, and JAK inhibitors and extend the interval of administration for biological drugs until nasopharyngeal swab PCR-SARS-CoV-2 tests results are negative or 3 after days of no fever and clinical improvement [194,195]. Since IBD patients were ruled out of the SARS-CoV-2 vaccine clinical trials, questions regarding the safety and effectiveness in patients with IBD are currently unanswered. As the COVID-19 vaccines available use mRNA-based technology (Pfizer/Biontech, ModeRNA) or non-replicating adenoviral vectors expressing the spike protein (AstraZeneca/Oxford, J&J, Sinovac, Sputnik V) the International Organization for the Study of Inflammatory Bowel Disease (IOIBD) recommends vaccinating all patients with IBD as soon as they are able to receive the vaccine, regardless of immune-modifying therapies[196]. The exception is for any live-attenuated virus vaccines or replication-competent viral vector vaccines that may come to market. Even The British Society of Gastroenterology agrees, strongly supporting SARS-CoV-2 vaccination in patients with IBD, underlining that the critical concerns in patients taking immunosuppressive drugs are more related to the risk of suboptimal immunization rather than the vaccine safety profile[197].

CONCLUSION

Due to the alterations in the immune response and the growing adoption of immunomodulators and biologics, IBD patients represent a population exposed to opportunistic infections. The risk of infections is also aggravated by an insufficient immunization status as too frequently observed in patients with IBD. In this field, gastroenterologists should be aware of viral infections that may complicate the IBD course, as they are often challenging to diagnose and treat. Moreover, excess hospitalizations due to viral infections and their related costs, contribute to the human and financial burden of IBD. Prevention is the most important step; a careful workup aimed at avoiding viral infectious disease is essential. Anamnestic and serological screening should be performed in all patients before starting immunosuppressive therapy. In addition, assessing and completing the vaccination status in IBD patients, ideally before immunosuppressive therapy, is the new standard of care. It is also important to provide prophylaxis when indicated to avoid the occurrence of potentially preventable infection. Continued vigilance and a high degree of suspicion is required to monitor for viral infection during maintenance therapy. Even if a direct correlation between a specific immunosuppressant drug and viral infection is not established, special attention must be paid to patients receiving corticosteroids, thiopurines, or combination therapy with thiopurines and anti-TNF agents as they are more prone to contracting opportunistic viral infections, in particular HSV, VZV, CMV, and EBV. It seems prudent to minimize the use of systemic steroids, think of alternatives to steroids and, if used, taper to the lowest possible dose quickly. As a matter of course, long-term steroids are contraindicated to avoid the risk of viral infection. Early diagnosis and appropriate treatment are necessary to prevent potentially severe complications that could precipitate the disease course if infective complications occur. Multidisciplinary cooperation between the gastroenterologist, infectious disease specialist and primary care physicians is the best way to optimize the care provided to IBD patients. The most significant gaps in knowledge in this area

are related to the difficulty in stratifying high-risk patients who may benefit from personalized preventive or therapeutic interventions. Prospective randomized controlled trials focused on IBD patients who are diagnosed with viral infection should be planned.

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REVIEW

Pancreatic cancer: A review of epidemiology, trend, and risk factors

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Abstract

Despite rapid advances in modern medical technology and significant improvements in survival rates of many cancers, pancreatic cancer is still a highly lethal gastrointestinal cancer with a low 5-year survival rate and difficulty in early detection. At present, the incidence and mortality of pancreatic cancer are increasing year by year worldwide, no matter in the United States, Europe, Japan, or China. Globally, the incidence of pancreatic cancer is projected to increase to 18.6 per 100000 in 2050, with the average annual growth of 1.1%, meaning that pancreatic cancer will pose a significant public health burden. Due to the special anatomical location of the pancreas, the development of pancreatic cancer is usually diagnosed at a late stage with obvious clinical symptoms. Therefore, a comprehensive understanding of the risk factors for pancreatic cancer is of great clinical significance for effective prevention of pancreatic cancer. In this paper, the epidemiological characteristics, developmental trends, and risk factors of pancreatic cancer are reviewed and analyzed in detail.

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Core Tip: Pancreatic cancer is still a highly lethal gastrointestinal cancer with a low 5-year survival rate and difficulty in early detection. A comprehensive understanding of the risk factors for pancreatic cancer is of great clinical significance for effective prevention of pancreatic cancer. In this review, the latest epidemiology, future trends, and various risk factors of pancreatic cancer are analyzed and summarized, which will provide more guidance and suggestions for the prevention and control of this malignancy.

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INTRODUCTION

The pancreas is an about 15-cm-long, spongy, tube-shaped organ located in the upper abdomen between the stomach and spine[1]. A normal healthy pancreas consists of acinar cells secreting digestive enzyme, ductal cells secreting bicarbonate, centroacinar cells that are the transitional region between acinar and ductal cells, endocrine islets secreting hormone, and relatively inactive stellate cells[2]. Pancreatic cancer occurs when abnormal DNA mutations in the pancreas cause pancreatic cells to uncontrollably grow and divide, forming tumors[3]. Pancreatic cancer is characterized as a fatal disease and one of the most aggressive and lethal malignancies[4,5]. By the time of diagnosis, pancreatic cancer often presents at an advanced stage, and has often spread to other parts of the body. Clinically, pancreatic cancer is the general term for malignant tumor formed in the epithelial cells of glandular structures in the pancreatic ductal cells, referred to as adenocarcinoma[6], and pancreatic ductal adenocarcinoma (PDAC) accounts for more than 90% of pancreatic cancers[7]. Due to the poor survival outcomes, PDAC is the seventh leading cause of global cancer death despite being the 10th most common cancer[8]. Other less common exocrine pancreatic cancers include adenosquamous carcinoma, squamous cell carcinoma, giant cell carcinoma, acinar cell carcinoma, and small cell carcinoma. At present, pancreatic cancer remains a devastating disease whose prognosis has remained largely unchanged over the last two decades[9]. Improvement in patient outcomes will depend on clear knowledge of epidemiology, reasonable prevention, and scientific regulation of early detection[4]. Therefore, it is necessary to understand the epidemiological characteristics, development trends, and risk factors of pancreatic cancer in detail, which will eventually establish rational prevention approaches for clinical benefit.

EPIDEMIOLOGY OF PANCREATIC CANCER

Assessing the latest epidemiologic trends in pancreatic cancer is necessary because it is of great importance for preventive measures and clinical care[10]. Therefore, we present a review of the latest epidemiology of pancreatic cancer.

Pancreatic cancer ranks consistently last among all cancers in terms of prognostic outcomes for patients and is predicted to become the second leading cause of cancer death in some regions[11]. A study including 84275 patients with at least 5 years of follow-up showed that actual 5-year survival rate in patients rose from 0.9% in 1975 to 4.2% in 2011 for all stages of pancreatic cancer, while in surgically resected patients, it increased from 1.5% to 17.4%[12]. In non-resected patients, the actual 5-year survival rate was 0.8% in 1975 and 0.9% in 2011, meaning that it remained roughly the same

between 1975 and 2011[12]. The 5-year relative survival rate of pancreatic cancer was 7.2% in China and the lowest level in all cancers[13]. Cancer Stat Facts showed that the 5-year survival rate at the time of diagnosis is approximately 10% in the United States based on data from Surveillance, Epidemiology, and End Results Program 18 between 2010 and 2016[14]. Pancreatic cancer has a poor 5-year survival rate, ranging from 2% to 9%, with little difference between high-income countries and low-income and middle-income countries[11,15]. Therefore, the 5-year survival rate of pancreatic cancer varies globally in different regions and countries, but does not exceed 10%. And it is predicted that patients with nonoperative pancreatic cancer have a lower 5-year survival rate.

According to Cancer Statistics 2021, the American Cancer Society reported approximately 60430 new cases and 48220 deaths for pancreatic cancer in the United States, ranking third after lung and bronchus cancer and colorectal cancer[16]. In the 28 countries of the European Union (EU), it was estimated that approximately 111500 people (55000 in males and 56500 in females) will die from pancreatic cancer by 2025, and the number of recorded deaths from the cancer in 2010 will increase by almost 50% (45% in men and 49% in women), and it has been projected that pancreatic cancer may become the third leading cause of cancer death in the EU after lung and colorectal cancers[17]. Global Cancer Statistics 2018 showed that the incidence and mortality of pancreatic cancer were 458918 and 432242 in 2018 in the world, respectively, and deaths account for about 94.2% of new cases[18]. Pancreatic cancer remains the seventh leading cause of cancer death globally, and Global Cancer Statistics 2020 showed that, globally, a total of 495773 new cases and 466003 related deaths were reported for pancreatic cancer in 2020, with almost as many mortality as incidence [19]. The systematic analysis for the 2017 Global Burden of Disease Study showed that the number of incident cases and deaths from pancreatic cancer in both genders increased 2.3-fold from 195000 incident cases and 196000 deaths in 1990 to 448000 incident cases and 441000 deaths in 2017 globally[15]. These reports indicate a gradual increase in the number of incident cases and deaths from pancreatic cancer.

Average age-standardized rates (ASRs) of pancreatic cancer incidence and mortality vary widely across regions of the world[19]. The ASR of the incidence was highest in Eastern Europe, with 9.9 per 100000, followed by Western Europe (9.8), Northern America (9.3), Southern Europe (8.4), Northern Europe (8.3), Australia/New Zealand (7.9), Micronesia/Polynesia (7.7), and Western and Eastern Asia (7.0)[19]. The ASR of the mortality was highest in Western Europe, with 7.4 per 100000, followed by Northern America (6.9), Northern Europe (6.7), Australia/New Zealand (6.7), Southern Europe (8.4), Eastern Europe (5.6), Eastern Asia (4.8), and Western Asia (4.4) [19]. The human development index (HDI) is a composite index that measures three dimensions: Life expectancy, education period, and access to essential sources for a suitable and reasonable life[20]. The ASRs of pancreatic cancer incidence and mortality in regions with a very high HDI were significantly higher than medium or low HDI regions[19]. The low ASRs of the incidence and mortality were found mainly in South-Central Asia (1.5 per 100000, 0.9 per 100000), Eastern Africa (2.0, 1.7), Middle Africa (2.0, 1.2), Western Africa (2.2, 1.8), Melanesia (2.9, 1.7), and South-Eastern Asia (2.9, 1.8), all of which are medium or low HDI regions[19]. The top six countries for pancreatic cancer incidence were Hungary (ASR, 11.2), Uruguay (ASR, 10.7), Japan (ASR, 9.9), Slovakia (ASR, 9.6), Czechia (ASR, 9.5), and Austria (ASR, 9.0), with 9.0 and greater per 100000, and a total of 21 countries, including the United States (ASR, 8.2), had an ASR of the incidence between 8.1 and 8.9 per 100000, as shown in Figure 1A [21]. The ASR of pancreatic cancer mortality was highest in Hungary and Uruguay, both at 10.2 per 100000, and a total of 26 countries, not including the United States (ASR, 6.6), had an ASR of the incidence between 7.2 and 8.6 per 100000, as shown in Figure 1B[21]. The proportion of estimated new cases for pancreatic cancer in China was relatively high in East China (9.4 per 100000), Northeast (9.4), Northwest (6.8), and North China (5.3), and was comparatively low in Central China (5.2), Southwest (4.3), and South China (3.6), having obvious regional characteristics[13]. Age-standardized rates of pancreatic cancer were 3-fold to 4-fold higher in higher HDI countries, compared with lower HDI countries[18]. The higher incidence and mortality rates of pancreatic cancer were reported in countries and regions with higher levels of HDI and Gross Domestic Product (GDP) per capita, and the coefficients of determination (R^2) of HDI and GPD per capita were high for the incidence and mortality[22]. The higher incidence and mortality rates of pancreatic cancer in countries with higher HDI indicates the importance that paying more attention and implementing appropriate programme to reduce risk factors acts as an effective measure to control the incidence and mortality of the cancer[23].

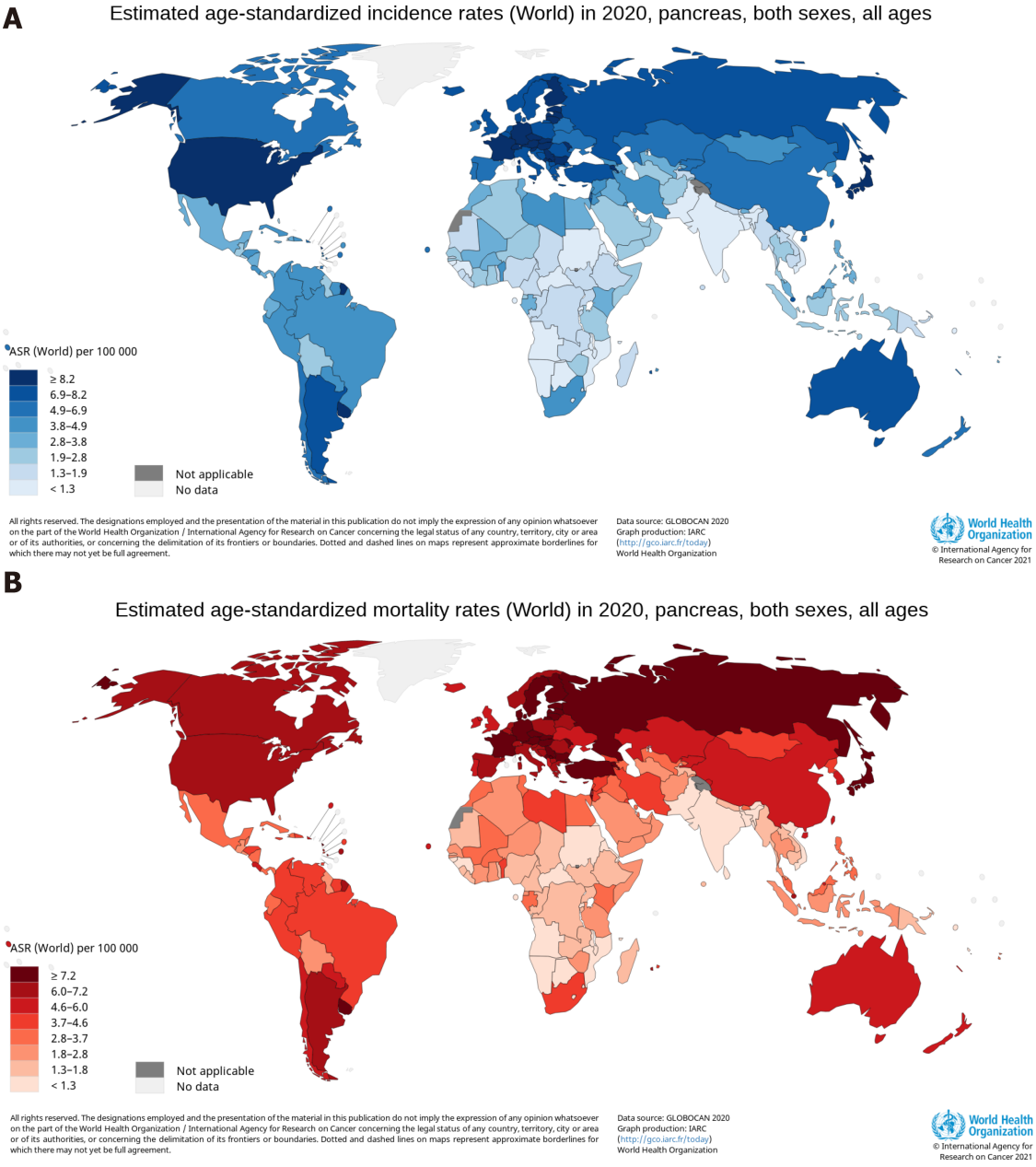


Figure 1 Maps showing estimated age-standardized rates of incidence and mortality for pancreatic cancer worldwide in 2020, including both sexes and all ages. A: Incidence; B: Mortality. Citation: Ferlay J, Ervik M, Lam F, Colombet M, Mery L, Piñeros M, Znaor A, Soerjomataram I, Bray F. Global Cancer Observatory: Cancer Today. [cited 20 Jan 2021]. In: International Agency for Research on Cancer [Internet]. Available from: https://gco.iarc.fr/today. Copyright ©International Agency for Research on Cancer 2021. Published by World Health Organization[21].

TRENDS OF PANCREATIC CANCER

Over the two decades from 2001 to 2020, the estimated new cases and deaths of pancreatic cancer have been increasing year by year in the United States, and the same trend has been observed among men and women, as shown in Figure 2. Using statistical models for analysis, in the United States, age-adjusted rates of new cases for pancreatic cancer remained stable from 2008 to 2017, and age-adjusted rates of death increased by an average of 0.3% each year from 2009 to 2018[14]. Prediction of pancreatic cancer incidence burden from the 28 member states of the EU and other selected countries around the world showed that in 2025, 2030, 2035, and 2040, the incidence will be 557688, 639030, 726740, and 815276, respectively, with growth rates of 21.5%, 39.2%, 58.4%, and 77.7%[24]. The incidence and mortality of pancreatic cancer in Africa will increase by 18327 and 17744 in 2040, respectively, with growth rates of 114.1% and 114.8%, the rates of which will be highest in the world, followed by Latin America and the Caribbean (incidence: + 99.3%; mortality: 101.0%)[25]. However, in 2040, the growth rates of the incidence and mortality in Europe will be

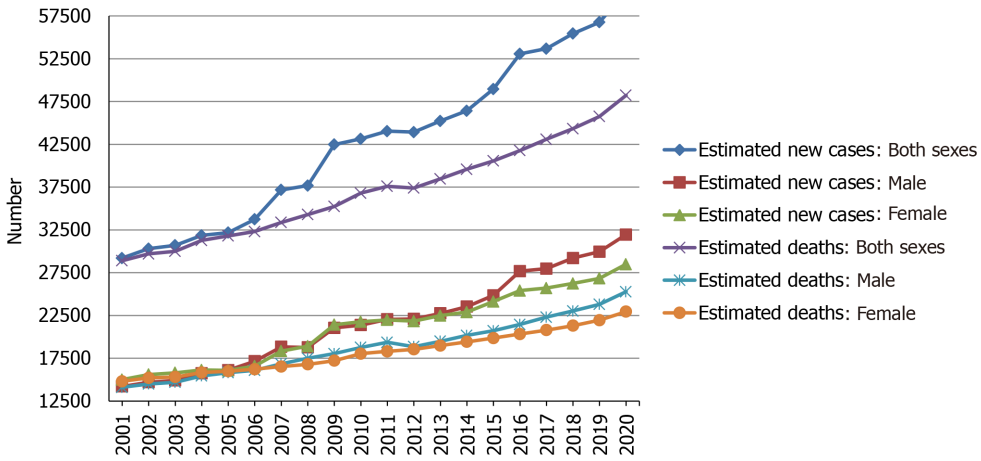


Figure 2 Estimated new cases and deaths from 2001 to 2020 in the United States. The data is from Cancer Statistics that the American Cancer Society estimates the numbers of new cancer cases and deaths in the United States from 2001 to 2020[16,28-47].

lowest at 29.3% and 31.6%, respectively[25]. Based on China and India, both countries in Asia with more than one billion population, the incidence and mortality in Asia will increase by 190532 and 182127 in 2040, respectively, which will be the largest increase in terms of number[25]. In addition, standardized mortality rate of pancreatic cancer increased from 1.30 per 100000 to 3.32 per 100000 over 1991-2014 and might reach the peak in the ensuing 5 years in China, and the mortality rate was higher among elderly people and in urban and northeast/eastern regions than among young people and in rural and middle/western regions[26]. The incidence of pancreatic cancer was 12.1 per 100000 in 2010 and is predicted to increase to 15.1 and 18.6 per 100000 in 2030 and 2050, respectively, with an average annual growth of 1.1%[27]. In the age-stratified analysis, the over 65 years group will have the highest projected incidence (31.9 per 100000) in 2050, and the incidence is projected to increase gradually in the sex-stratified analysis, with an average annual growth of 1.3% in males and 0.9% in females[27].

The number of years of life lost (YLL) is a measure of premature mortality, taking into account simultaneously the number of deaths and life expectancy at age of death, and projection of YLL due to premature mortality (*i.e.*, time-based approach) provides a comprehensive outlook of the fatal burden at a population level[27]. Due to premature death in individuals with pancreatic cancer, the total YLL was 5604 years in 2010 and is projected to increase to 9784 in 2030 and 14247 in 2050, with an average annual growth of 2.1%[27]. In the age-stratified analysis, the 40-64 years group will have the highest projected YLL (7588 years) in 2050, and the YLL is projected to increase gradually in the sex-stratified analysis, with an average annual growth of 2.1% in males and 2.2% in females[27].

In conclusion, pancreatic cancer, like other cancers such as lung, liver, and stomach cancer, will cause a huge economic burden to all countries and related populations in the next 20 years, especially China having a huge population, which is still a developing country. In order to reverse these trends and improve the prognosis of patients with pancreatic cancer, the most simple, direct, and effective way is to understand the risk factors affecting the occurrence and development of pancreatic cancer in detail, which provides comprehensive and reasonable guidance and suggestions for the prevention of pancreatic cancer, and offers reliable and feasible ideas for the early screening of pancreatic cancer.

CAUSES AND RISK FACTORS OF PANCREATIC CANCER

Pancreatic intraepithelial neoplasias (PanINs) are noninvasive epithelial proliferations in smaller pancreatic ducts, which progress from PanIN-1 (low-grade) to PanIN-2 (intermediate-grade) to PanIN-3 (high-grade)[48]. The differentiation of normal epithelium into PanIN-1/PanIN-2 and then into PanIN-3/invasive pancreatic cancer requires a considerable period of development, while the development process before high-grade PanIN-3 and invasive pancreatic cancer is the golden stage of preventing pancreatic cancer through effective interventions. Therefore, a thorough and compre-

hensive understanding of pancreatic cancer risk factors is of great practical significance for the prevention of pancreatic cancer. The exact cause of pancreatic cancer is unknown, but many non-modifiable and modifiable risk factors are associated with development of pancreatic cancer. Non-modifiable risk factors include age, gender, ethnicity, ABO blood group, microbiota, diabetes mellitus (DM), and family history and genetic susceptibility, while modifiable risk factors include smoking, alcohol drinking, dietary factors, pancreatitis, obesity, infection, and socioeconomic status and insurance. The influence of these factors on the occurrence, progression, and invasion of pancreatic cancer is analyzed and summarized as follows.

NON-MODIFIABLE RISK FACTORS

Age

Both 89.4% of new cases of pancreatic cancer and 92.6% of deaths occur in patients over 55 years of age in the United States, the new cases are most frequently diagnosed among people 65-74 years of age with a median age at diagnosis of 70 years, and the percent of deaths is also highest among people of the same age group with a median age at death of 72 years[14]. The proportions at 40-64 years and over 65 years of age were 47.9% and 48.6% in diagnosed patients with pancreatic cancer in China[49]. The mortality rates of patients aged under 30, 30-44, 45-59, 60-74, and 75 and above among males are 0.1, 1.4, 10.1, 19.3, and 14.6 per 100000 in China, respectively[50], meaning that male pancreatic cancer population over 60 years of age has a higher mortality rate. The reference does not provide the mortality rates of pancreatic cancer in the five broad age groups in women and both sexes[50]. Worldwide, it is extremely rare for pancreatic cancer to be diagnosed before the age of 30, so it is typically a disease of the elderly. The risk factor also determines the need for screening and early detection of pancreatic cancer among the population over a certain age.

Gender

In the United States, the new cases of pancreatic cancer is 31950 among males and 28480 among females in 2020, and the deaths is 25270 among males and 22950 among females[16]. In China, the age-standardized respective incidence and mortality rate are 52.2 and 45.6 per 100000 among men in 2015, and 37.9 and 33.8 per 100000 among women[50]. On a global scale, the new cases are 243033 among men and 215885 among women in 2018, and the deaths are 226910 among men and 205332 among women[18]. The global respective incidence and mortality rates are 5.5 and 5.1 per 100000 among men in 2018, and 4.0 and 3.8 per 100000 among women[18]. Globally, the respective cumulative risk of developing pancreatic cancer and dying from it from birth to 74 years is 0.65% and 0.59% among males in 2018, and 0.45% and 0.41% among females [18]. The ratio of male to female for estimated new cases and deaths increased in the United States from 2001 to 2020, as shown in Figure 3. Thus, the worldwide incidence and mortality of pancreatic cancer are higher among males than females.

ABO blood group

The ABO antigens were first described by Landsteiner as erythrocyte antigens in 1900 [51]. The antigens of ABO blood group are glycoproteins that are expressed on red blood cells and various epithelial cells, including the urothelium and gastrointestinal mucosa[52]. The phenotypic A and B antigens are terminal carbohydrates synthesized by the addition of monosaccharides catalyzed by a series of specific glycosyltransferases, and the phenotype O is characterized by deficiency of A and B glycosyltransferases[52]. There is growing evidence that ABO blood group may also be associated with carcinogenesis or progression of pancreatic cancer. A consortia-based evaluation and replication study showed that non-O blood group was associated with an increased risk of pancreatic cancer compared with blood group O[53]. A register-based cohort study showed that blood group A was associated with an increased risk of pancreatic cancer[54]. There was a significantly higher risk for developing pancreatic cancer in Chinese patients with the A or AB blood types than for those with type O [55]. El Jellas *et al*[56] reported that the prevalence of blood type A and subtype A1 was highest among the unresected cases, the unresected cases had the lowest frequency of blood group O, and patients with blood group O survived longer than non-O patients in the group of unresected cases. A study at Shanghai Pancreatic Cancer Institute showed that Chinese Han population with blood type A were more likely to develop pancreatic cancer, but people with blood type B were less likely to develop pancreatic neuroendocrine tumors and other types of pancreatic masses, compared with those

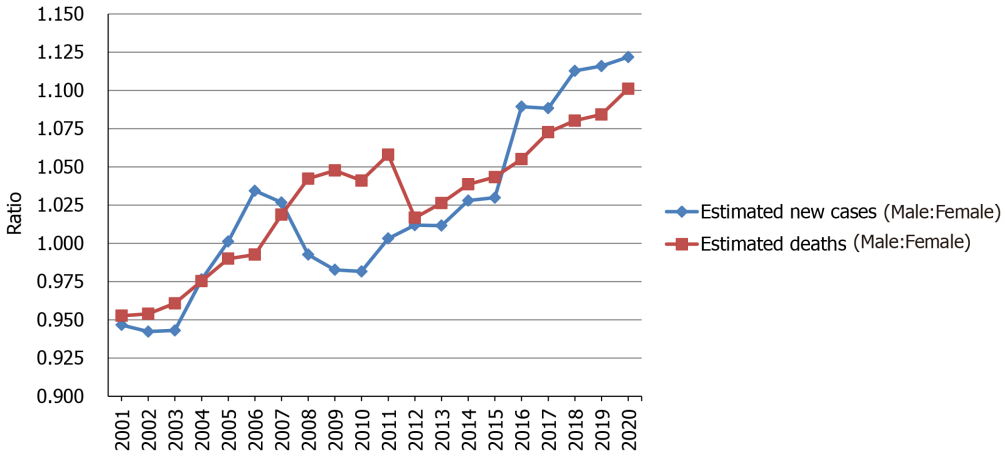


Figure 3 Ratio of male to female for estimated new cases and deaths from 2001 to 2020 in the United States. The data is from Cancer Statistics that the American Cancer Society estimates the numbers of new cancer cases and deaths in the United States from 2001 to 2020[16,28-47].

with blood type O[57]. Hofmann *et al*[51] reported that patients with blood type O had more often well-differentiated PDAC compared with blood type non-O, and they elucidated the novel interaction between blood type immunoglobulin M isoagglutinins and PDAC O-GalNAc glycoproteins, which may contribute to the pathogenesis and progression of pancreatic cancer. Accordingly, the risk for people with blood type O to develop pancreatic cancer is lower than those with other blood types. In addition, the ABO allele that determines blood type A has two major subtypes, namely, A1 and A2, and the association of A1 but not A2 with pancreatic cancer could therefore suggest that the activity of blood type A glycosyltransferase plays a role in carcinogenesis[56]. The study showed that the A2 subtype has a single base deletion near the carboxyl terminal, and introducing the single base deletion into the expression construct of A1 transferase cDNA significantly reduced the activity of A transferase in DNA-transfected HeLa cells[58]. Therefore, clarifying the etiological mechanism between the risk of pancreatic cancer and ABO blood type may provide a new perspective for the treatment of this disease.

Ethnicity

The burden of exocrine pancreatic disease, including pancreatic cancer, pancreatitis, and pancreatic cyst, differs among various ethnicities, and African-Americans and certain indigenous populations are at the greatest risk of developing these diseases [59]. Huang *et al*[60] observed that African-Americans, Native Americans, and Japanese-Americans had higher rates of developing pancreatic cancer, but no difference between Latino- and European-Americans, and found that African-Americans had a 20% greater risk of pancreatic cancer than European-Americans even after adjusting for known risk factors. In the studies including few minority patients, the neutrophil-to-lymphocyte ratio (NLR) is associated with a reduced overall survival in pancreatic cancer patients, and NLR > 5 was significantly associated with a worse overall survival compared with NLR ≤ 5[61]. Patients with an NLR ≤ 5 were also more likely to develop locally advanced disease than metastatic cancers and primary tumor located in the head or neck of the pancreas, while patients with an NLR > 5 were more likely to have liver metastases and albumin < 3.4 g/dL, suggesting that elevated NLR is an independent marker for poor prognosis and a potentially valuable factor[61]. Patients with an NLR ≤ 5 were more likely to be non-Hispanic Black, while patients with an NLR > 5 were more likely to be non-Hispanic White or Hispanic[61], suggesting that there are different predispositions and outcomes for pancreatic cancer between non-Hispanic Black and non-Hispanic White or Hispanic. Gad *et al*[62] found that the incidence of pancreatic cancer among Asian-Americans, especially malignancies of the body and tail of the pancreas, as well as the mortality based on the incidence, was overall on the rise in an epidemiological study, without respect to age, sex, or stage subgroup. Amaral *et al*[63] also emphasized the importance of the influence of ethnicity on somatic mutations in Brazilian patients with PDAC. To elucidate the reasons for the racial differences in the incidence of pancreatic cancer may help us improve the understanding and prevention of this disease.

Microbiota

Oral microbiota: Several epidemiological studies have found the direct relationship between oral bacteria and pancreatic cancer[64]. Farrell *et al*[65] reported that the levels of two bacteria biomarkers (*Neisseria elongate* and *Streptococcus mitis*) were lower in patients with pancreatic cancer than in healthy controls, and found that the combination of the two bacteria biomarkers distinguished pancreatic cancer patients from healthy subjects with an area under the curve value of 0.90, sensitivity of 96.4%, and specificity of 82.1%. Torres *et al*[66] found that the ratio of *Leptotrichia* to *Porphyromonas* in the saliva of pancreatic cancer patients was significantly higher than that in healthy individuals or those with other disease. Fan *et al*[67] found that *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* were associated with a higher risk of pancreatic cancer, and phylum Fusobacteria and its genus *Leptotrichia* were associated with a decreased risk of this cancer. Olson *et al*[68] reported that the mean relative proportions of Firmicutes and related taxa were higher in patients with pancreatic cancer, while the mean relative proportions of Proteobacteria and related taxa were higher in controls.

Gut microbiota: Studies have confirmed that gut microbiota is associated with recognized risk factors for pancreatic cancer, such as obesity and type II diabetes, suggesting the relationship between gut bacteria and pancreatic cancer[64]. In recent decades, multiple and highly complex effects of gut microbiota on pancreatic cancer have been identified as potential risk factors for the development and progression of this tumor[69]. A prospective study, for the first time, analyzed gut microbial profile in Chinese pancreatic cancer cohorts by MiSeq sequencing, revealing a significant decline in gut microbial diversity and a unique microbial profile in pancreatic cancer, due in part to the decline in alpha diversity[70]. Additionally, the microbial profile changed in pancreatic cancer, with an increase in certain pathogens and lipopolysaccharides (LPS)-producing bacteria and a decrease in probiotics and butyrate-producing bacteria [70]. LPS might play a pro-inflammatory pro-tumor role by activating the nuclear factor-κappa B (NF-κB) pathway, producing proinflammatory cytokines [tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, and IL-1] and leading to liver inflammatory and oxidative damage[71]. After LPS treatment, Ras activity in cells prepared from acinar-Ras mice was greatly elevated and maintained at a high level for a long time, and severe chronic pancreatitis and PanIN lesions were induced in acinar-Ras mice, accompanied by sustained elevated Ras activity, whereas there was no observed effects in control mice, suggesting that LPS treatments led to fibrosis and PanIN formation in the presence of oncogenic Ras[72]. Therefore, LPS may have a greater pathological impact on patients carrying cells expressing oncogenic RAS, which may explain individual differences in response to infection, suggesting the association between chronic bacterial infectious diseases and colon and pancreatic cancers[72]. As a member of the RAS family of GTP-binding proteins, KRAS mediates a wide variety of cellular functions including proliferation, differentiation, and survival[73], and the prevalence of oncogenic *KRAS* mutation in PDAC ranges from 88% to 100%[74]. Consistent with a central pathogenic role of the $KRAS^{G12D}$ mutation, mice engineered with pancreas-specific expression of this activated *KRAS* allele sustain classical PanIN lesions that can progress to PDAC in the appropriate tumor suppressor background [73]. Thomas *et al*[75] found that the proportion of poorly differentiated PDAC in the microbiota-intact mice was higher than that in microbiota-depleted mice (89.75 *vs* 34.8%, respectively), demonstrated that the intestinal microbiota accelerated pancreatic carcinogenesis in the $KRAS^{G12D}/PTEN^{lox/+}$ mice model of pancreatic cancer, and considered that the intestinal microbiota had a long-distance role on PDAC progression. In addition, based on crucial genera associated with pancreatic cancer, gut microbial markers might achieve an excellent classification capacity between pancreatic cancer and healthy controls, suggesting that the specific alterations of gut microbiota might become non-invasive biomarkers for pancreatic cancer diagnosis[70].

Pancreatic microbiota: A number of different bacterial taxa are found in pancreatic tissue contents, including those known to inhabit the oral cavity, which suggest that the pancreas is not a sterile organ[76]. The relative abundance of bacterial taxa at the genus level in the pancreas has a substantial between-person variability, and bacterial composition of the pancreatic duct, head, and tail as well as the duodenum was highly similar in the same individuals[76]. The study showed that the community composition of the microbiota in the human pancreas failed to discriminate between normal and disease states, and that the acquisition of pancreatic bacteria is not a physiologic process, even under conditions of intestinal inflammation[75]. Del Castillo *et al*[76] found that the presence and relative abundance of *Lactobacillus* were lower in

pancreatic tissue of cancer subjects and the relative abundance of periodontal-related pathogens was higher in cancer subjects, when compared with noncancer subjects. Pushalkar *et al*[77] found that the bacteria (*Enterococcus faecalis* and *Escherichia coli*) could migrate into the pancreas, confirmed that the abundance of intrapancreatic bacteria in both mice and patients with PDAC was markedly greater compared with normal pancreas by 16S rRNA fluorescence *in situ* hybridization and qPCR analysis, and consider that the bacteria promote the progression of pancreatic oncogenesis in both preinvasive and invasive models[77]. Maekawa *et al*[78] mainly detected *Enterococcus* and *Enterobacter* species in bile, demonstrating that *Enterococcus* and *Enterobacter* can survive in pancreatic juice and/or bile, and found that 29 of 36 pancreatic juice samples were positive for bacterial DNA[78]. *Enterococcus faecalis* was also found in pancreatic tissue from patients with chronic pancreatitis and pancreatic cancer, and serum antibodies to capsular polysaccharide of *Enterococcus faecalis* were elevated in patients with chronic pancreatitis[78]. They demonstrated that *Enterococcus faecalis* is involved in the progression of chronic pancreatitis using the model mice with caerulein-induced chronic pancreatitis, which may ultimately result in the development of pancreatic cancer[78].

In addition, Pushalkar *et al*[77] also found that the microbiome regulates immunogenicity in PDAC and programs tumor-associated macrophages *via* Toll-like receptor signaling to induce immune tolerance, and bacterial communities are distinct between early and advanced PDAC. Geller *et al*[79] demonstrated that bacteria are a component of the PDAC tumor microenvironment, and estimated that bacteria colonized PDAC samples had an average of one bacterium per 146 human cells. Their results indicated that PDACs contain bacteria that can potentially modulate tumor sensitivity to gemcitabine, and they considered that the bacteria play a critical role in mediating resistance to chemotherapy[79]. By studying colon cancer models, they found that bacteria can metabolize the chemotherapeutic drug gemcitabine (2′,2′-difluorodeoxycytidine) into its inactive form (2′,2′-difluorodeoxyuridine), which depends on the expression of a long isoform of bacterial cytidine deaminase, seen primarily in γ-proteobacteria[79].

The above various microorganisms have different influences on the occurrence, development, and invasion of pancreatic cancer in different ways. Therefore, the study on the influence of microorganisms on pancreatic cancer will provide more new insights to reveal its etiology. And studying the effects of microorganisms on pancreatic cancer has the potential to be used as a target for the regulation of disease progression and treatment.

Family history and genetic susceptibility

Hereditary pancreatic cancer includes inherited cancer syndromes with a recognized known germline mutation associated with an increased risk of pancreatic cancer and familial pancreatic cancer with two or more cases of pancreatic cancer in their families [80]. Pancreatic cancer associated with hereditary syndromes or familial pancreatic cancer accounts for about 10% of cases[81]. Family history of pancreatic cancer was associated with an increased pancreatic cancer risk when compared with cancer-free family history, with the risk being greater when ≥ 2 first-degree relatives suffered pancreatic cancer and among current smokers[82]. Members of familial pancreatic cancer kindreds having at least one first-degree relative affected by pancreatic cancer had a 9-fold increased risk of developing pancreatic cancer, whereas members of sporadic pancreatic cancer kindreds having a first-degree relative with pancreatic cancer did not have an increased risk[83]. Risk was higher among members of familial pancreatic cancer kindreds with a young-onset patient (< 50 years) in the kindred than those without a young-onset case in the kindred[84].

Genetic mutations associated with an increased risk of pancreatic cancer include *STK11/LKB1*, *CDKN2A (p16)*, *BRCA1/2*, *PRSS1/SPINK1/CFTR*, mismatch repair genes (*MLH1/MSH6/MSH2/PMS2*), *ATM*, and *PALB2* (a new pancreatic cancer susceptibility gene)[85]. And pancreatic cancer is also found to be associated with these familial cancer syndromes for which genetic mutations correspond, such as Peutz-Jeghers syndrome (*STK11/LKB1*), familial atypical multiple mole melanoma (*CDKN2A*), hereditary breast cancer ovarian cancer syndrome (*BRCA1/2*), and hereditary non-polyposis colorectal carcinoma syndrome (*MLH1/MSH6/MSH2/PMS2*)[25,85].

A study from Mayo Clinic showed that the aggregate prevalence was 36/302 (11.9%) for all cases with any positive PDAC family history[86]. Seven PDAC-associated genes (*ATM*, *BRCA1*, *BRCA2*, *CDKN2A*, *MSH2*, *PALB2*, and *PMS2*) and four genes with no known PDAC association (*BARD1*, *CHEK2*, *MUTYH/MUY*, and *NBN*) were identified as pathogenic variants in the study[86]. Kindreds with at least one pair

of first-degree relatives who were affected by PDAC were considered FPC, and kindreds with at least two affected blood relatives that did not meet the FPC definition were considered "familial non-FPC"[86]. Thirty-six (12%) patients carried at least one pathogenic variant in one of 11 genes, and the probabilities of carriers with pathogenic variant among FPC patients and familial non-FPC patients were 14% and 9%, respectively[86]. Pathogenic variants (n) identified in PDAC patients were *BRCA2* (11), *ATM* (8), *CDKN2A* (4), *CHEK2* (4), *MUTYH/MYH* (3 heterozygotes, not biallelic), *BRCA1* (2), and 1 each in *BARD1*, *MSH2*, *NBN*, *PALB2*, and *PMS2*[86]. Regardless of FPC status, multiple susceptibility gene testing may be necessary in PDAC patients with a family history of pancreatic cancer, which will provide genetic risk counseling for families[86]. Therefore, the study of the family characteristics and genetic features of pancreatic cancer is of great clinical significance in identifying the susceptible population of pancreatic cancer, screening the high-risk individuals of pancreatic cancer, and early diagnosis of pancreatic cancer.

Diabetes mellitus

In comparison with patients without diabetes, those who were recently diagnosed with diabetes had an nearly 7-fold increase in risk of developing pancreatic cancer [87]. Either hyperglycaemia or diabetes is found among as many as 80% of patients, both of which can be detected in the presymptomatic phase, and on the contrary, older patients with new-onset diabetes have about an 8-fold higher risk of developing pancreatic cancer than the general population, suggesting a "dual causality" between diabetes and PDAC[88], in that both long-standing type 2 DM (T2DM) is a risk factor of developing PDAC and PDAC is assumed to be a cause of diabetes in many cases [89]. A multiethnic cohort study also showed that recent-onset diabetes is a manifestation of pancreatic cancer and long-standing diabetes is a risk factor of developing this cancer[90]. At present, the prevalence of diabetes in China is on the rise and has the largest diabetes epidemic worldwide, and in 2013, the estimated total prevalence was 10.9% for diabetes and 35.7% for prediabetes among adults in China, indicating the importance of diabetes as a public health problem in China[91]. In 2011-2012, the prevalence of diabetes was estimated from 12% to 14% among US adults, and participants who were non-Hispanic black, non-Hispanic Asian, and Hispanic had a higher prevalence[92]. The global spread of this enormous medical burden further highlights the necessity to better understand the pathophysiological relationship between T2DM and pancreatic cancer.

A growing body of epidemiological and experimental evidence shows that chronic hyperinsulinaemia increases the risk of cancers of the colon and endometrium, and probably other tumours (such as pancreas and kidney)[93]. Hyperinsulinemia, especially intrapancreatic, due to obesity and insulin resistance in patients with prediabetes or early T2DM may believably conduce to the observed increased risk of developing PDAC[89]. The high level of islet hormones in blood directly reaches groups of acinar and ductal cells and acts on insulin-like growth factor-1 (IGF-1) receptors to promote survival and proliferation of acinar and ductal cells[89]. The characteristics of T2DM patients and the overwhelming majority of obese individuals are insulin resistance with ensuing hyperinsulinemia and high levels of IGF-1, which can act as potent growth-promoting factors[94]. In addition, Butler *et al*[95] reported that replication of pancreatic duct cells in lean subjects with T2DM had a 4-fold increase compared with lean non-diabetic controls, suggesting that the increased risk of pancreatitis and pancreatic cancer in T2DM is driven by replication of chronically increased pancreatic duct cell replication[95].

The desmoplastic response attribute to production and proliferation of extracellular matrix proteins in tumor-associated fibroblasts, activating pancreatic stellate cells (PaSC)[94]. Yang *et al*[96] reported that progression of high-fat diet-induced PDAC in mice is associated with hyperglycemia, hyperinsulinemia, and PaSC activation, and found that the pancreas from patients with T2DM showed substantial collagen deposition and activated PaSC in islet and peri-islet exocrine pancreas compared with normal control[96]. Both quiescent and activated PaSC coexpress insulin and IGF-1 receptors, the expression of which was modulated by both insulin and glucose[96]. Insulin induces rapid tyrosine autophosphorylation of insulin/IGF-1 receptors at specific kinase domain activation loop sites, activates Akt/mTOR/p70S6K signaling, and inactivates FoxO1, a transcription factor suppressing cell growth[96]. In activated PaSC, insulin promotes cell proliferation and production of extracellular matrix proteins, and specific inhibition of mTORC1 and mTORC2 can abolish the above effects, suggesting that increased local glucose and insulin concentrations are associated with obesity and T2DM promotes PaSC growth and fibrosing responses [96]. In premalignant H6c7-kras cells, hyperglycemia increases secretion and signaling

of transforming growth factor beta1 (TGF-β1) and induces properties of cancer stem cells depending on TGF-β1-signaling, suggesting that hyperglycemia promotes pancreatic ductal epithelial cells to acquire the properties of mesenchymal and cancer-stem cells by activating TGF-β signaling[97]. Li *et al* found that patients with A blood type who also had DM had a greater odds of having pancreatic cancer, and further research is needed to confirm the results and to identify the mechanisms by which A blood type and DM jointly contribute to the risk of pancreatic cancer progression and development[55].

There are many mechanisms that explain the effect of DM on pancreatic cancer and the relationship between the two. However, in order to reveal the real relationship between diabetes and pancreatic cancer, it still needs to be further studied to provide new strategies for the prevention of pancreatic cancer.

MODIFIABLE RISK FACTORS

Smoking

Epidemiological studies have shown that many causative factors are associated with pancreatic cancer, and cigarette smoking has the strongest positive association with the risk of developing the cancer[98]. Due to smoking, the estimated prevalence was about 30% in many parts of the world and the risk of pancreatic cancer was doubled in smokers, whereas the population-attributable risk caused by smoking is about 25% for pancreatic cancer, meaning that the overall burden of this cancer would be reduced if smoking was completely eliminated[99]. Patients with pancreatic cancer who smoked prior to diagnosis had an about 40% increased hazard for death compared with those who never smoked[100]. And long-term smoking portended worse outcomes for current smokers, but former smokers experienced outcomes similar to those who had never smoked, suggesting that quitting smoking can have potential beneficial effects [100]. A large European case-control study confirmed that current smokers had a 72% increased risk of developing pancreatic cancer compared with never-smokers, and the study also endorsed that around 16% of all pancreatic cancer diagnoses could be avoided through tobacco preventive measures in terms of attributable risk[101]. And analysis of dose-response relationships confirmed that higher smoking intensity, longer smoking duration, and increased cumulative dose levels were associated with a further increased risk of pancreatic cancer, whereas smoking cessation led to a gradual decline in the risk of pancreatic cancer[101]. Smoking also notably increases the risk of developing pancreatic cancer in individuals with a family history of this cancer[82]. These studies suggest that smoking cessation has a potential benefit to improve survival for patients with pancreatic cancer and helpfully prevent pancreatic cancer in those at risk.

As an avoidable risk factor, smoking is of particular concern, and elucidating the mechanisms involved would significantly reduce the number of PDAC cases diagnosed each year[102]. Smoking-induced inflammation was accompanied by enhanced activation of PaSC and elevated levels of serum retinoic acid-binding protein 4, suggesting increased bioavailability of retinoic acid that is conducive to differentiation of myeloid-derived suppressor cells to tumor-associated macrophages and dendritic cells[103]. And smoking exposure also leads to partial suppression of the immune system in the early progression of pancreatic cancer[103]. In xenografts of patient-derived pancreatic cancer, nicotine intervention promoted growth and metastasis of tumor, and it was confirmed that nicotine reduced survival by enhancing paracrine HGF-MET signaling in the pancreatic cancer microenvironment[104]. In addition, nicotine induced dedifferentiation of acinar cells by activating AKT-ERK-MYC signaling, thereby inhibiting the activity of *Gata6* promoter and losing GATA6 protein, and subsequently causing loss of acinar differentiation and over-activation of oncogenic K-Ras[105]. And metformin could inhibit nicotine-induced carcinogenesis of the pancreas and tumor growth by up-regulating GATA6 expression and promoting programmed differentiation of acinar cell[105]. Benzo(a)pyrenes, polycyclic aromatic hydrocarbons, and tobacco-specific nitrosamines are several carcinogens identified in tobacco smoke, most of which play a genotoxic role by formation of DNA adducts and generation of reactive oxygen species, leading to mutations in vital genes such as *K-Ras* and *p53*[106]. Nicotine and other carcinogenic components in tobacco smoke can directly promote growth of tumor cells, change cross-talk between tumor and stromal cells within the tumor microenvironment, and enhance infiltration of myeloid-derived suppressor cells[100]. Therefore, the study and elucidation of carcinogenic mechanism of carcinogens in tobacco smoke will contribute to the treatment and prevention of

pancreatic cancer caused by related factors.

Alcohol drinking

East Asians have a high proportion in inefficient metabolism of acetaldehyde, so alcohol drinking may play a more important role in the developing pancreatic cancer among East Asians[107]. According to many studies, there is no doubt that the risk of pancreatic cancer is associated with high alcohol consumption (more than three drinks per day), but no association was found with low-to-moderate alcohol consumption [25]. A population-based study demonstrated that heavy alcohol consumption and binge drinking increased estimated risk of developing pancreatic cancer among males but not among females[108]. It may also be the reason why pancreatic cancer has a higher incidence and mortality in men than in women. And the study also suggested that either binge or consistent heavy alcohol consumption persistently increased the risk of developing pancreatic cancer regardless of the temporal proximity between alcohol consumption and diagnosis of pancreatic cancer[108]. A large prospective study suggested that baseline and lifetime alcohol consumption was positively associated with the risk of developing pancreatic cancer, and the estimated risk for beer and spirits/liquors was more apparent than wine[109]. Alcohol plays an independent role in promoting PDAC associated with fibrosis formed by a stellate cell-independent mechanism and further boosts formation of PanIN lesion and induction of M2 macrophages in the context of chronic pancreatitis[110]. This is an important finding, namely, M2 macrophages suppress the directed immune mechanisms of cancer and block the recruitment of T cells into the tumor, further promoting cancer progression[111]. Mice that expressed mutant *K-ras* gene developed early and advanced forms of the most common pancreatic cancer in humans[112]. Specific mutations in the *K-ras* oncogene may be more commonly found in alcohol consumers with pancreatic cancer, and may be initiators or terminators of pancreas cancer associated with heavy alcohol consumption[108]. Additionally, alcohol might promote the development of cancer by inducing oxidative stress and lipid peroxidation, and alcohol abuse may also accelerate the progression of tumor by boosting pancreatic inflammation[112].

Two NAD-dependent enzymes, namely, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), are mainly involved in alcohol metabolism in human[113]. In the body, alcohol is first converted into acetaldehyde by ADH oxidation, and acetaldehyde is then converted into non-toxic acetate by ALDH oxidation for excretion[113]. In the human liver, there are two kind of ALDHs, including ALDH1 (cytosolic) and ALDH2 (mitochondrial), and only mitochondrial ALDH2 oxidizes acetaldehyde to acetate at physiological concentrations[114]. The variant *ALDH2*2* allele can significantly decrease ALDH2 enzyme activity and affect alcohol response, which is a striking genetic polymorphism[113].

In many East Asian countries, the *ALDH2*2* allele associated with reduced enzyme activity is found in 30%-50% of the population, resulting in the inefficient metabolism of the carcinogenic acetaldehyde generated from alcohol metabolism[107]. Thus, acetaldehyde accumulates in *ALDH2*2* carriers, even after a moderate intake of ethanol (0.5 g/kg)[115], also leading to a higher risk of developing alcohol-related cancers in individuals carrying the *ALDH2*2* allele. Kanda *et al*[116] found that the impact of alcohol on pancreatic cancer risk was associated with rapid production or high accumulation of acetaldehyde, indicating that acetaldehyde may play a substantial role in the potential mechanism of pancreatic cancer[116]. This can also provide guidance and help to prevent pancreatic cancer for people of different groups and races.

Dietary factors

In general, diets high in fruits, vegetables, and other plant-based foods can reduce the risk of developing pancreatic cancer, while dietary patterns rich in meat and animal products can increase the risk of the cancer[107]. Intake of meat, especially red meat, cooked meat at high temperature, and meat-associated heterocyclic amines, and overall mutagenic activity may induce the development of exocrine pancreatic cancer [117]. Foods are often heat processed and may contain advanced glycation end products (AGEs) that can be formed by a nonenzymatic reaction between reducing sugars and free amino groups on proteins, peptides, and amino acids[118]. When the carbonyl group of reducing sugar like glucose or its oxidation or lipid peroxidation products react with the ε-amino group of lysine or the guanidino group of arginine, AGEs can be formed, including imidazolones, pentosidine, pyrraline, and N^{ε}-(carboxymethyl)lysine (CML)[118]. AGEs accumulate in sera and tissues during the ageing

process because of glycolytic and oxidative reactions, reduced activity of the detoxification systems, cigarette smoking, and consumption of high-temperature-processed foods[8]. Excessively high concentrations of AGEs in human tissue and circulation accelerate oxidative stress and inflammation, which may play a pathogenic role[118]. Exogenous AGEs, in particular those derived from the diet, have been claimed to contribute to several disease processes, including cancer in general and, specifically PDAC[8]. CML is frequently used as a marker for AGEs in general[119]. Jiao *et al*[120] proposed a novel mechanism that consumption of heat-treated red meat can cause chronic inflammation and subsequently lead to pancreatic cancer, and considered that consumption of dietary CML-AGE was associated with a modest increase in risk of pancreatic cancer for men, which might partly explain the positive correlation between red meat and pancreatic cancer. A model experiment showed that AGEs markedly accelerated the development of pancreatic cancer and inhibition of AGE prevented the tumor-promoting effect of diabetes[8]. Therefore, exogenous AGEs from processed/grilled/baked foods may be involved in the genesis and development of pancreatic cancer.

Many nutrients and phytochemicals in fruits and vegetables have antioxidant, antimutagenic, and anti-carcinogenic properties, especially for water-soluble vitamins [121]. Additionally, isothiocyanates found in cruciferous vegetables powerfully induce the detoxification enzymes, and assist in the removal of potential carcinogens[121]. Higher intake of fruit and vegetables is associated with a reduced risk of pancreatic cancer[122], and cruciferous vegetable intake might be inversely associated with pancreatic cancer risk[123]. A meta-analysis of epidemiological studies showed that high intake of dietary fiber was associated with a risk reduction of pancreatic cancer [124]. A European Prospective Investigation Into Cancer-Norfolk study showed that high-fiber diet altered a positive correlation between red/processed meats and the PDAC development but not those with lower fiber intake, and fiber intake made few alteration for the PDAC risk in past and current smokers[125]. This study showed that fiber intake may be beneficial for those with high meat intake, but the findings do not suggest that high fiber intake can protect against the PDAC development[125]. Therefore, further prospective cohort studies are needed to investigate the effect of fiber intake on the development and progression of pancreatic cancer. A low-fat dietary intervention was associated with a reduced incidence of pancreatic cancer in overweight or obese women in the Women's Health Initiative Dietary Modification trial[126]. Therefore, it is of great significance for the prevention of pancreatic cancer by adjusting the diet of pancreatic cancer susceptible population. And it is worth further investigating how both diet and lifestyle may work together to promote or inhibit pancreatic cancer.

Pancreatitis

The majority of burden in exocrine pancreatic disease arises from acute pancreatitis, chronic pancreatitis, and pancreatic cancer[127]. Acute pancreatitis, an inflammatory disease of exocrine pancreas, is associated with injury and necrosis of tissue[128]. It has been reported that acute pancreatitis may be an early symptom of pancreatic cancer[129]. A nationwide matched-cohort study in Denmark showed that patients hospitalized with acute pancreatitis had an increased risk of developing pancreatic cancer compared with age- and gender-matched controls in the general population [130]. Rijkers *et al*[131] found that patients who suffered a first incident of acute pancreatitis and had no further progression to chronic pancreatitis had a 0.4% risk of developing pancreatic cancer, but the risk of pancreatic cancer increased 9-fold for those who progressed to chronic pancreatitis, suggesting that screening for pancreatic cancer after a first incident of acute pancreatitis, especially in patients who had further progression to chronic pancreatitis, could potentially result in more curable resections and improved survival[131]. The risk of pancreatic cancer increased markedly after an initial diagnosis of acute pancreatitis, regardless of its type, and gradually decreases with passage of time[132]. An increase in the number of recurrent episodes of acute pancreatitis was associated with an increased risk of developing pancreatic cancer [132].

Patients who have an episode of acute pancreatitis are 20%-30% more likely to have one or more relapse, and approximately 10% of the relapsing cases progress to chronic pancreatitis[133]. Chronic pancreatitis is a progressive inflammatory disease, and causes pancreatic parenchyma to be replaced by fibrous tissue, resulting in a loss of acinar and islet cells[134]. Chronic pancreatitis may cause mild or asymptomatic debilitating pain, attack(s) of acute pancreatitis, endocrine and/or exocrine deficiency, local and/or systemic complications, and pancreatic cancer[135]. In recent decades, there is accumulating evidence that longstanding pre-existing chronic pancreatitis is a

strong risk factor of developing pancreatic cancer[25]. Low body mass index and pancreatic exocrine insufficiency in patients with chronic pancreatitis define a high-risk population with latent PDAC[136]. In comparison with the general population, patients with chronic pancreatitis had a significantly increased risk of pancreatic cancer, especially those with an older age at onset and a > 60 pack-year smoking history[137]. Five years after diagnosis, the risk of pancreatic cancer increased nearly 8-fold in patients with chronic pancreatitis, but the association diminishes with long-term follow-up[138]. The same risk trend was observed in patients with recurrent acute pancreatitis and chronic pancreatitis, suggesting the need for close follow-up in the first few years after diagnosis of these two types of pancreatitis to avoid neglect of pancreatic cancer. Patients who need surgery to treat chronic pancreatitis have a very high risk of developing pancreatic cancer, and early surgical intervention can play a role in preventing the progression of chronic pancreatitis to pancreatic cancer[139]. Chronic pancreatitis patients with *de novo* postoperative diabetes have a high suspicion index of developing pancreatic cancer after surgery[139].

Patients with early-onset pancreatitis caused by genetic factors appear to have a higher risk of developing pancreatic cancer[140]. Mutations of susceptibility genes in chronic pancreatitis can determine hereditary pancreatitis, idiopathic chronic pancreatitis, and cystic fibrosis, and Cazacu *et al*[140] found that mutations of cystic fibrosis transmembrane conductance regulator (*CFTR*) genes modestly increase the risk of pancreatic cancer in a meta-analysis. A total of 50080 patients were diagnosed with pancreatic cancer, of which 14.8% (7420 cases) were diagnosed with idiopathic pancreatitis prior to the diagnosis of cancer[141]. After pancreatitis diagnosis, six risk factors significantly associated with pancreatic cancer diagnosis included age between 40 and 90 years, African-American race, male sex, smoking, obesity, and DM, suggesting that it may be warranted to screen patients older than 40 years with unclear etiology of pancreatitis, especially for African-Americans and male population[141].

The study of the promoting effect of pancreatitis on the development of pancreatic cancer is beneficial to the early detection of pancreatic cancer, and can provide more guidance for the prevention of pancreatic cancer. However, in order to better understand the promoting role of pancreatitis on pancreatic cancer, more studies are needed to clarify the mechanism of pancreatitis in the development of pancreatic cancer.

Obesity

Obesity has been more and more recognized as a strong but modifiable risk factor for pancreatic cancer[142]. Relevant studies have confirmed that obesity is associated with an increased incidence of pancreatic cancer and potentially worse outcomes of this cancer[142]. A cohort study with pooled analysis found that central obesity was associated with increased mortality of pancreatic cancer, independent of body mass index, and also suggested that being overweight or obese during early adulthood may have a significant impact on the mortality risk of pancreatic cancer later in life[143]. A nationwide study including 1.79 million Israeli adolescents showed that obesity (≥ 95th percentile) was associated with an increased risk of pancreatic cancer later in life among both men and women compared with normal weight (5th to- < 85th percentile)[144].

There have been two biological mechanisms that were proposed to explain the underlying association between obesity and risk of pancreatic cancer, including inflammation and hormonal misbalance[142]. Many human cancers result directly from chronic inflammation, and inflammation has emerged to be a key mediator of pancreatic cancer development[145]. Changes in the fibro-inflammatory microenvironment are the major feature of obesity-associated pancreatic tumors[146]. Obesity is a pro-inflammatory condition, and both hypertrophied adipocytes and immune cells (primarily lymphocytes and macrophages) residing in adipose tissue contribute to increased circulating levels of pro-inflammatory cytokines like TNF-α, IL-6, leptin, and adiponectin[147]. The imbalance between these finely regulated pro-inflammatory and anti-inflammatory bioactive molecules leads to changes in tissue microenvironment, which further have influence on cell proliferation, apoptosis, cell invasion, and angiogenesis[148]. For example, Hertzer *et al*[149] reported that conditional *KRAS*^{G12D} mice with a high fat, high calorie diet exhibited significantly increased inflammation in the peri-pancreatic fat accompanied by elevated levels of several inflammatory cytokines, such as IL6, IL13, and IFN-γ, suggesting that obesity-associated inflammation in peri-pancreatic fat may accelerate pancreatic neoplasia in the model mice[149].

Obesity is also often associated with insulin resistance and T2DM, along with raised levels of insulin and IGF-1[142]. Insulin resistance is a hallmark of T2DM, in which

insulin fails to trigger adequate glucose uptake, resulting in accumulation of glucose in bloodstream and raised levels of insulin[150]. Hyperglycemia can enhance the availability of nutrients to cancer cells which metabolize glucose through the Warburg effect[151]. Islet adaptation enhances hormone production, processing, and secretion in the setting of obesity[146]. Even moderate overall and abdominal obesity and weight gain during adulthood were independently associated with an increased risk of developing hyperinsulinemia in non-diabetic middle-aged men[152]. Hyperinsulinemia causes a rise of IGF-1 which activates PI3K/MAPK/mTOR pathways after binding with its receptor, or the IGF receptor[148]. Overactivation of these pathways can activate the Ras/ERK pathway, leading to an increase in cell division, and IGF-1 activating PI3K/AKT/mTOR pathways promotes proliferation and inhibits apoptosis [142].

The mutation of oncogenic KRAS is the major event in pancreatic cancer and permanently activates KRAS protein, and then the protein serves on a molecular switch to activate various signaling pathways and transcription factors in cells, inducing cell proliferation, migration, transformation, and survival[153]. In comparison with lean KC mice, the pancreas of obese KC mice showed an increase in activation of KRAS downstream pathways, including MAPK and PI3K/AKT/mTORC1[154]. Chung *et al*[146] found that β-cell aberrantly expressed peptide hormone cholecystokinin in response to obesity and showed that islet cholecystokinin promoted oncogenic KRAS-driven tumorigenesis in pancreatic duct.

Infection

Helicobacter pylori (*H. pylori*) found mainly in the stomach is a Gram-negative microaerophilic pathogen that chronically infects as much as half the world's population[155]. *H. pylori* infection is associated with a variety of malignancies, such as gastric cancer, premalignant lesions of the stomach (atrophic gastritis and intestinal metaplasia), gastric lymphoma, pancreatic cancer, colorectal cancer, and laryngeal cancer[156]. With an estimated prevalence between 25% and 50% in Westernized countries, *H. pylori* could result in 4% to 25% of all cases with pancreatic cancer in these countries[157]. *H. pylori* infection is closely, *albeit* weakly, associated with the development of pancreatic cancer, and the association is prominent in Europe and East Asia, but less so in North America[158]. Cytotoxin-associated antigen A (CagA), a 120-145-kDa protein, was for the first time described as a virulence factor of *H. pylori* related to peptic ulcers[159]. A risk of pancreatic cancer increased in individuals with seropositivity for CagA-negative *H. pylori*, whereas the risk decreased in individuals with seropositivity for CagA-positive *H. pylori*[160]. CagA-negative strains of *H. pylori* might be a causative factor of pancreatic cancer[161]. Xiao *et al*[158] reported that CagA-positive *H. pylori* strains appear not to be associated with pancreatic cancer.

The net effects of *H. pylori* colonization in the gastric antrum are paracrine disinhibition of antral G-cell function, hypergastrinemia, and hyperacidity[162]. The risk of pancreatic cancer is increased by long-term conditions of excess gastric/duodenal acidity[162]. Gastric acid drives pancreatic bicarbonate secretion and, a consequence of hyperchlorhydria and suppressed somatostatin increases bicarbonate output from the pancreas in *H. pylori* carriers[162]. Low-level, prolonged generating of secretin or pancreatic bicarbonate increases the activity and turnover rate of ductular epithelial cell to sufficiently enhance the carcinogenic effect of environmental or endogenous N-nitroso carcinogens[162]. Asymptomatic *H. pylori* colonization, non-ulcer dyspepsia, or duodenal ulcers, and exposure to N-nitroso carcinogens *via* dietary or other routes in individuals would increase the risk of developing pancreatic cancer by increasing basal secretors or pancreatic bicarbonate[162]. CagA injected into gastric parietal cells through the interaction of *H. pylori* with integrin results in the activation of extracellular regulated protein kinases (ERK1/2) to further mobilize NF-κB p50 homodimers into the nucleus, leading to the inhibition of gastric H,K-adenosine triphosphatase (H,K-ATPase) α subunit transcription and the repression of gastric acid secretion[163]. Seropositivity of CagA-positive *H. pylori* was shown to protect against pancreatic cancer when compared to CagA-negative *H. pylori*, suggesting that differential modification of CagA-negative *vs* CagA-positive strains of *H. pylori* on chronic gastric acidity may be involved in modulating the risk of pancreatic cancer[160].

Socioeconomic status and insurance

In contrast with the traditional biomedical model, the bio-psycho-social-medical model highlights the significant role of socioeconomic status in health care services, including insurance status, marital status, and poverty level[164]. A study suggested that African-Americans, and in some cases Hispanics, had lower rates of surgery, less accessed to aggressive stage specific treatment, and underwent surgery at low volume

hospitals and/or by lower volume surgeons, which might contribute to the differences in outcomes[165]. In addition, underinsured or uninsured patients also tended to receive less aggressive treatment[165]. A study of a total of 83902 patients with pancreatic cancer showed that patients with lower socioeconomic status were less likely to undergo surgical resection among patients with localized/regional pancreatic cancer[166]. Among patients with localized/regional pancreatic cancer who underwent surgical resection, patients with higher socioeconomic status have better overall survival, and patients with lower socioeconomic status have worse pancreatic cancer-specific survival compared with patients with higher socioeconomic status[166]. These findings suggest that racial differences in treatment and outcomes might be attributable to socioeconomic, insurance, and geographic factors[165]. The study of pancreatic cancer cases from the National Cancer Database from 2004 to 2015 showed that private insurance was associated with more treatment and better survival, higher education was associated with earlier treatment, and treatment was less and delayed among African-Americans despite later diagnosis[167]. After adjusting for socioeconomic status, African-Americans had about the same rate of survival overall at integrated facilities and the survival was improved, suggesting that higher socioeconomic status was associated with better treatment and survival[167]. A pan-cancer analysis showed that socioeconomic status was strongly associated with 1-mo postoperative mortality in primary solid tumors, and the risk of dying was high within 1-mo after surgery in socioeconomically disadvantaged people[164]. And underserved populations around the world often face similar barriers to cancer treatment, largely reflecting inequalities in social factors[165]. Therefore, socioeconomic status plays an extremely important role in the prevention and prognosis of pancreatic cancer, and the formulation of policies targeting low socioeconomic status patients may improve the low 5-year survival rate of pancreatic cancer.

CONCLUSION

Over the next 10 years to 20 years, an increase in pancreatic cancer is inevitable. At the same time, in the face of the characteristics of high mortality and difficult early diagnosis of pancreatic cancer, early prevention of pancreatic cancer through understanding the risk factors for pancreatic cancer is an economical and effective means, which is to prevent pancreatic cancer in advance. In view of the non-modifiable factors affecting pancreatic cancer, we may screen the susceptible population to pancreatic cancer, and provide reliable screening strategies and reasonable diagnostic ideas for the early diagnosis of pancreatic cancer. By studying the modifiable risk factors that affect pancreatic cancer, we may provide earlier interventions to prevent pancreatic cancer so that it can be possibly blocked in its early stages of canceration, thus significantly reducing the incidence of pancreatic cancer. Globally, a comprehensive prevention and control strategy for pancreatic cancer should include effective tobacco-control policy, recommendations for healthier lifestyles, and enlarging coverage of screening, education and vaccination programmes to better improve public awareness of the need to take precautions.

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REVIEW

Minimally invasive image-guided therapy of primary and metastatic pancreatic cancer

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Abstract

Pancreatic cancer is a challenging malignancy with limited treatment options and poor life expectancy. The only curative option is surgical resection, but only 15%-20% of patients are resectable at presentation because more than 50% of patients has distant metastasis at diagnosis and the rest of them has locally advanced pancreatic cancer (LAPC). The standard of care first line treatment for LAPC patients is chemotherapy with or without radiation therapy. Recent developments in minimally invasive ablative techniques may add to the treatment armamentarium of LAPC. There are increasing number of studies evaluating these novel ablative techniques, including radiofrequency ablation, microwave ablation, cryoablation and irreversible electroporation. Most studies which included pancreatic tumor ablation, demonstrated improved overall survival in LAPC patients. However, the exact protocols are yet to set up to which stage of the treatment algorithm ablative techniques can be added and in what kind of treatment combinations. Patients with metastatic pancreatic cancer has dismal prognosis with 5-year survival is only 3%. The most common metastatic site is the liver as 90% of pancreatic cancer patients develop liver metastasis. Chemotherapy is the primary treatment option for patients with metastatic pancreatic cancer. However, when the tumor is not responding to chemotherapy or severe drug toxicity develops, locoregional liver-directed therapies can provide an opportunity to control intrahepatic disease progression and improve survival in selected patients. During the last decade new therapeutic options arose with the advancement of minimally invasive technologies to treat pancreatic cancer patients. These new therapies have been a topic of increasing interest due to the severe prognostic implications of locally advanced and metastatic pancreatic

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cancer and the low comorbid risk of these procedures. This review summarizes new ablative options for patients with LAPC and percutaneous liver-directed therapies for patients with liver-dominant metastatic disease.

Key Words: Pancreatic neoplasms; Interventional radiology; Ablation techniques; Electroporation; Radiofrequency ablation; Therapeutic embolization

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Core Tip: During the last decade new therapeutic options arose with the advancement of minimally invasive technologies to treat pancreatic cancer patients. These new therapies have been a topic of increasing interest due to the severe prognostic implications of pancreatic cancer and the low comorbid risk of these procedures. This review summarizes new ablative options for patients with locally advanced pancreatic cancer and percutaneous liver-directed therapies for patients with liver-dominant metastatic disease.

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INTRODUCTION

Pancreatic cancer carries an extremely poor prognosis with the 5-year survival is around 8% and goes down to 3% in patients with metastatic disease[1]. Although the overall cancer-survival statistics are improving, pancreatic cancer is an exception with no major therapeutic advancement in the last 30 years since the introduction of pancreaticoduodenectomy[2].

As the survival statistics of other malignancies are improving, it is expected that pancreatic cancer becomes the second leading cause of cancer-related death by 2030[3].

The only curative option of pancreatic cancer is complete surgical resection. However, only 15%-20% of patients have potentially resectable disease at presentation [4] approximately 30% patients are unresectable due to locally advanced pancreatic cancer (LAPC)[5] and approximately 50% of patients have stage IV pancreatic cancer with distant metastasis at diagnosis[1]. The most common metastatic site is the liver as 90% of pancreatic cancer patients develop liver metastasis[6].

During the last decade new therapeutic options arose with the advancement of minimally invasive technologies. This review summarizes new ablative options for patients with LAPC and percutaneous liver-directed therapies for patients with liver-dominant metastatic disease. The term pancreatic cancer includes a histologically heterogenous group. This article focuses only on pancreatic ductal adenocarcinoma, as the most common malignancy of the pancreas, accounting for 85%-95% of cases[4,5].

MINIMALLY INVASIVE TREATMENTS OF LAPC

Non-metastatic LAPC comprises 30% of all newly diagnosed pancreatic cancer cases [7]. Patients with LAPC are not surgical candidates because of unresectable involvement of adjacent vessels like the portal vein, celiac or superior mesenteric artery or their major branches. Current standard-of-care therapy of LAPC is chemotherapy with or without radiation therapy. Adjuvant chemotherapy can downstage 20% of LAPC patients to be potentially resectable. Downstaged, surgically resected patients had a significantly improved survival (35.3 mo) compared to those who did not became surgical candidate after chemotherapy (16.2 mo)[8].

Patients with LAPC who do not became surgical candidate, may benefit from image-guided local ablation therapies, either percutaneously or during intraoperative open approach. The ablation technologies used in patients with LAPC include heat-based ablations, like radiofrequency ablation (RFA), microwave ablation (MWA) and

cryoablation and the new non-thermal ablation technique, irreversible electroporation (IRE).

RFA of LAPC

RFA is the most widely used heat-based ablative method which utilizes high frequency alternating electric current that causes cell death by heating the tissue through rapid electron vibration generating frictional heat[9]. This method of heat generation means that RFA is heavily dependent on conductivity which has close correlation to the water content of the tissue[9,10]. This is one of the disadvantages of RFA because as the tissue adjacent to the electrode heats up, it becomes desiccated and then acts as an "insulating sleeve" around the ablation probe hindering electron flow and further heat generation, thus limiting the ablation zone size[9]. Another major factor which limits the extent of the ablation zone is the cooling effect of flowing blood which works as a "heat sink"[11].

RFA treatment of the primary pancreatic cancer usually performed during open laparotomy. The studies where pancreatic RFA was used during open laparotomy are listed in Table 1. The initial publication by Matsui *et al*[12] included 20 patients and demonstrated that RFA is feasible in pancreatic cancer. This milestone publication was followed by multiple studies using RFA in pancreatic cancer with different endpoints. Wu *et al*[13] studied the pain relief effect of RFA and reported 50% pain reduction after pancreas cancer RFA. Girelli *et al*[14] also reported significantly reduced pain score in 69% of symptomatic patients. Several studies evaluated overall survival following RFA of pancreatic cancer and reported overall survival from 14.7 mo up to 33 mo[15,16]. The largest study[16] included 107 patients and divided patients into 2 arms: 47 patients underwent RFA as a first-line treatment and 60 patients received neoadjuvant therapy first followed by RFA. All patients received standard of care post-RFA treatment which included systemic chemotherapy and/or radiation and 29 patients also received intra-arterial chemotherapy which consisted of injection of epirubicin and cisplatin into the celiac artery in every 4 wk until disease progression. Median overall survival was 14.7 mo in the RFA group and 25.6 mo in the neoadjuvant treatment + RFA group. They also reported that 32 patients treated with the triple combination of RFA, radiation therapy, and intraarterial chemotherapy had an even longer median survival of 34 mo.

There is only one publication reporting data of percutaneous RFA of the pancreas tumor[17]. The authors analyzed data of 23 patients who underwent ultrasonography (US)-guided percutaneous RFA. There was no severe adverse event reported, which may be explained by the fact that in all patients the tumor located in the pancreatic tail or body and none in the head of the pancreas. Follow-up imaging showed good response to RFA, however, the median overall survival or pancreatic progression-free survival was not reported.

While RFA of pancreatic cancer has proven survival benefit, it also comes with a high morbidity risk of up to 40%[14] and mortality rate of up to 25%[13]. The most frequent major complications are portal vein stenosis (15%) and heat-injury of the duodenum (8%)[14]. The reported most common cause of death is massive gastrointestinal hemorrhage[13]. Girelli *et al*[18] reported that avoiding temperatures to exceed 90 °C during ablation and preserving surrounding tissue from overheating can reduce complications.

Despite the reported potential survival benefit of RFA, it remains unclear when RFA is best incorporated into the first line standard of care systemic chemotherapy/chemoradiation treatment protocol; should RFA performed before or after the standard of care systemic therapy? Does its potential high complication rates worth its benefit?

MWA of LAPC

MWA is another heat-based ablation technology which appears to have several advantages over RFA. Microwave generates heat using electromagnetic radiation-induced rotation of dipole molecules, such as water[19]. MWA is more powerful than RFA and generates higher temperatures in a shorter time. This leads to larger ablation zones and less heat-sink effect from the adjacent blood vessels[20]. MWA can be effective in tissues with high impedance, such as charred desiccated tissue, what is a weakness of RFA[20].

The available data on the usage of MWA in LAPC is very limited[21-24]; only 4 studies have been published with limited follow-up and survival data included (Table 2). The largest series of 20 patients reported 100% technical success without major complications and only 9.8% minor complications. However, 3-mo follow-up imaging was available in 10 patients (50%) only and no overall survival was reported

Table 1 Studies evaluating intraoperative pancreas radiofrequency ablation

Ref.	Study type	Patients	Outcome measure	Results	Complica-tions
Giardino *et al*[16], 2013	Retrospective	107	OS	RFA 1st line: 14.7 mo RFA + adjuvant: 25.6 mo	Mortality: 1.8%; morbidity: 28%
Girelli *et al*[18], 2013	Prospective	100	OS and DSS	20 and 23 mo	Mortality 3%; morbidity 24%
Girelli *et al*[14], 2010	Prospective	50	Safety and feasibility	-	Mortality 2%; morbidity 24%
Spiliotis *et al*[15], 2007	Retrospective	25	OS	OS: 33 mo[1]	Mortality: 0%; morbidity: 23%
Wu *et al*[13], 2006	Prospective	16	Pain relief	50% pain relief	90-d mortality: 25%;
Matsui *et al*[12], 2000	Prospective	29	OS	OS: 3 mo	Mortality: 10%

[1]Mean value. OS: Overall survival; DFS: Disease-free survival; PFS: Progression-free survival; DSS: Disease-specific survival.

Table 2 Studies evaluating microwave ablation of pancreatic cancer

Ref.	Patients	Survival	Procedure	Guidance	Major adverse events
Lygidakis *et al*[23], 2007	15	Up to 22 mo	Open	N/A	No
Carrafiello *et al*[21], 2013	10	80% at 1 yr	5 open, 5 percutaneous	US, US/CT	2
Vogl *et al*[24], 2018	20	N/A	Percutaneous	CT	No
Ierardi *et al*[22], 2018	5	N/A	Percutaneous	US	No

US: Ultrasound; CT: Computed tomography.

[24]. In the study of Lygidakis *et al*[23] 15 patients were included with large pancreatic tumors (average tumor size of 6 cm) and only partial ablation was achieved in all patients. No major complications were reported. The longest follow-up was 22 mo; survival data of the group was not published. Carrafiello *et al*[21] reported of 10 patients with LAPC who underwent MWA; 5 during open surgery, and 5 percutaneously. Two major complications were reported, one pancreatic pseudocyst requiring drainage and one arterial pseudoaneurysm. The 1-year survival rate was 80%. Ierardi reported 100% technical success in 5 patients without major complications and 60% partial response and 40% progressive disease at 1 mo follow-up[22].

In our experience of 2 patients with pancreatic tail cancer, MWA was very effective without major complications; in one patient the ablation was complete (Figure 1), in the other patient the tumor recurred in the pancreatic tail 17 mo after ablation. Besides the recurrence, the MWA was also complicated with a development of an asymptomatic pancreatic pseudocyst which required no treatment. The local recurrence was first treated with stereotactic body radiation therapy which provided local control with stable disease for approximately a year, then the patient underwent repeat MWA of the enlarging pancreatic recurrence and a new solitary liver metastasis 32 mo after the initial ablation. There was no evidence of recurrence at the latest follow-up 6 mo after the second MWA.

Cryoablation of LAPC

During cryoablation intra- and extracellular ice crystals causing damage to the cell membrane, that will lead to cell death due to dehydration and osmotic pressure changes[25]. The cooling mechanism of the cryoablation probe based on the Joule-Thomson effect as high pressure argon gas is circulated *via* tiny tubes inside the probe with sudden expansion of the gas in a chamber in the ablation probe tip. The probe tip reaches temperatures as low as -160 C. The major advantage of cryoablation over other ablation technologies is that ice is visible on US, computed tomography (CT) and magnetic resonance imaging, therefore, the size of the iceball can be monitored during the ablation to prevent inadvertent damage to adjacent tissues. The other advantage is the analgesic property of cold during cryoablation which is associated with reduced intra- and postprocedural pain[26,27]. The heat sink effect of adjacent vessels can negatively influence the size of the ablation zone in cryoablation, but this effect is not as pronounced compared to RFA[28]. One of the potential major complications of cryoablation is cryoshock which occur in 0.3 to 2.0% of patients[29,30] and is charac-

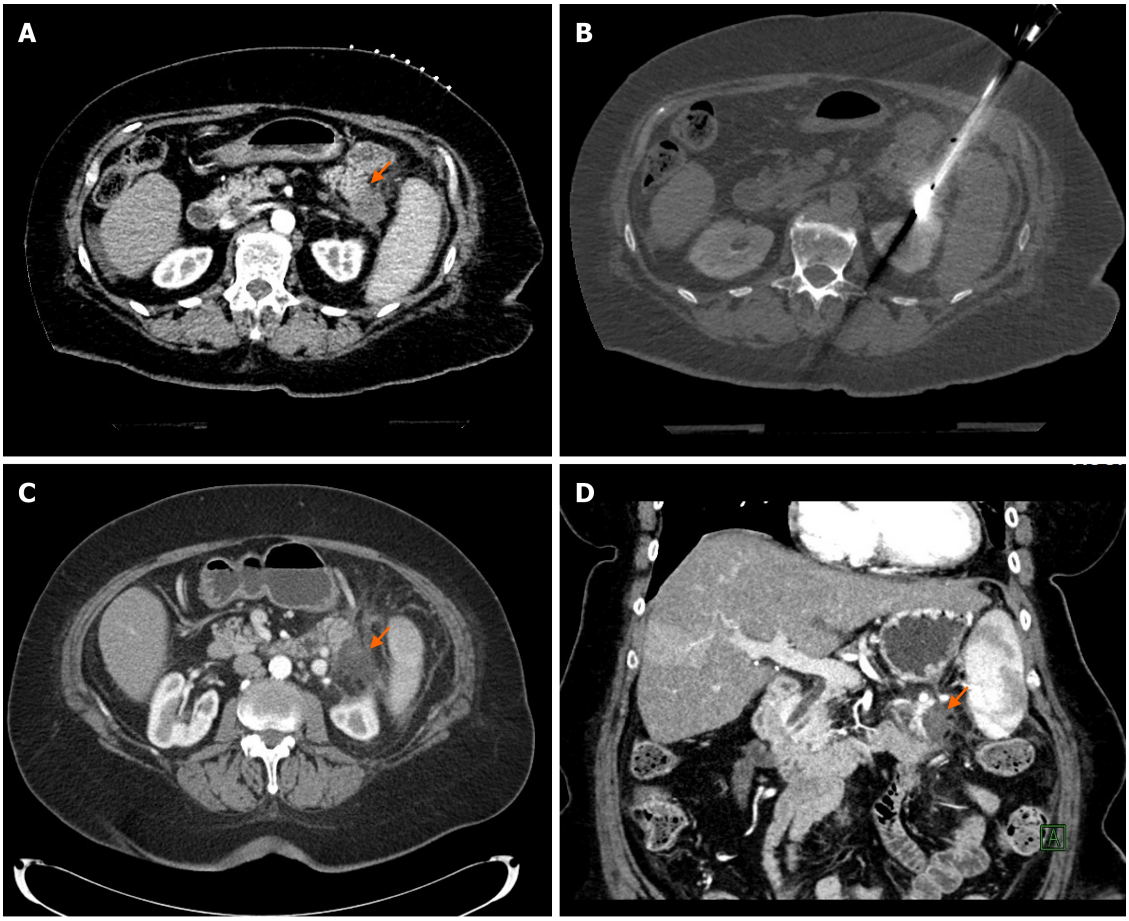


Figure 1 Percutaneous computed tomography-guided microwave ablation of a pancreatic tail adenocarcinoma. A: 72-year-old patient presented with a 1.9 cm biopsy-proven pancreatic adenocarcinoma in the tail of the pancreas as it shown on this contrast-enhanced computed tomography (CT) image (orange arrow); B: The patient was not surgical candidate due to comorbidities and underwent percutaneous CT-guided microwave ablation (MWA) of the pancreatic tumor. CT image shows the microwave antenna in place in the pancreatic tail tumor; C and D: Axial and coronal contrast-enhanced CT images 6 wk after the MWA show good radiological response with an avascular area in the tail of the pancreas corresponding to the ablation zone (orange arrows). Patient developed peritoneal metastases 4 mo later and died 13 mo after the MWA treatment.

terized by multiorgan failure and disseminated intravascular coagulation[31]. Cryoshock was observed most often in large volume liver ablations[32].

Similar to MWA, the available data on cryoablation of pancreatic cancer is very limited. There is no data regarding the effect of cryoablation in patients with LAPC. There are 3 papers which reported pancreatic cryoablation, but two of them included patients with stage 4 pancreatic cancer[33,34] the third study used combination of cryoablation with radioactive iodine-125 treatment[35]. Song *et al*[33] compared gastrojejunal bypass surgery alone *vs* bypass surgery with pancreatic tumor cryoablation in 118 patients with advanced pancreatic cancer. Cryoablation was performed in 42 patients. There were no differences between the patient characteristics and postoperative mortality; however, the 1-year survival rate was superior in the cryoablation group (4.8% *vs* 2.6%). The reason for the poor overall survival rate is the inclusion of patients with distant metastases.

Niu *et al*[34] compared 4 groups of patients with metastatic pancreatic cancer who underwent cryotherapy alone (36 patients), immunotherapy alone (17 patients), chemotherapy alone (22 patients) or cryoimmunotherapy (31 patients). Cryotherapy was used to treat both the primary tumor and the metastatic sites. Median overall survival (OS) was significantly higher in the cryoimmunotherapy (13 mo) group compared to cryotherapy (7 mo), immunotherapy (5 mo) and chemotherapy (3.5 mo) groups. There was no major complication.

Xu *et al*[36] reported outcomes of 49 patients of whom 38 underwent a combination therapy of cryoablation and iodine-125 seed implantation for LAPC. Both laparotomic and percutaneous approach were used in this study. Complete response was seen in 20.4% of patients, partial response in 38.8%, stable disease in 30.6% and progressive disease in 10.2%. The median OS was 16.2 mo, and the 12-mo and 24-mo survival was 63.1% and 22.8%, respectively. Six patients (12.2%) developed acute pancreatitis, in

one case it was considered as severe.

Given the promising results with cryoablation, further studies are warranted in patients with LAPC.

IRE of LAPC

Irreversible electroporation is a novel ablation technology[36], which, unlike RFA, MWA, and cryoablation, is non-thermal. IRE delivers high-voltage electrical current (1500 to 3000 V) between the IRE ablation probes which creates nanoscale defects in the cell membranes[37-39]. The cells within the ablation zone lose the ability to maintain homeostasis which results in apoptotic cell death with narrow zone of transition[36-38]. Since this is a non-thermal ablation, its efficacy is independent of thermal conductivity of the tissues and not influenced by the "heat-sink" effect of adjacent vessels[36,39]. The ablation zone is very predictable and easy to monitor using US or CT[39]. The high voltage delivered by IRE may cause muscular contraction and cardiac arrhythmia. Therefore, IRE must be performed under general anesthesia with complete neuromuscular blockade and electrocardiogram synchronization. Patients with pacemakers may not be a candidate for IRE[40].

The main advantage of IRE is the controlled apoptosis of the cells without harming the adjacent collagen matrix and proteins, thus saving scaffolds of blood vessels and bile ducts[41-44]. As IRE leads to apoptotic cell death it enhances antigen presentation to immunocompetent cells which may improve immunological response locally and at abscopal metastatic sites. Irreversible nanopore formation at the ablation site and reversible nanopore formation in the vicinity of the ablation zone can lead to higher intracellular concentration of chemotherapeutic agents in the damaged cancer cells[45, 46]. The disadvantage of IRE is that it requires placements of multiple ablation probes because the high electric currents delivered between the probes. The probes have to be parallel to each other and at the same tissue depth which adds complexity to the probe placements and increases procedure time.

IRE most commonly performed intraoperatively during open laparotomy (Table 3, Figure 2). Although, some surgeons use palpation and visual guidance for ablation probe placements, most commonly intraoperative US is used to precise identification of the tumor extent and visualization of major arteries inside the tumor to maximize ablation success and minimize bleeding complications.

Several studies described survival benefit for patients who underwent IRE (Table 3) [47-50]. Huang *et al*[47] found that median OS was significantly extended to 22.6 mo. The authors analyzed data of 70 patients who were treated with IRE for LAPC. All patients received neoadjuvant chemotherapy with either a gemcitabine-based or TS-1 (tegafur, gimeracil, and oteracil) chemotherapy based on the patient's age and performance status. Local recurrence rate was only 8.6% at 28 mo follow-up. Disease progression was noted in 25 patients (36%) at distant sites, mostly in the liver (12 patients). Both median OS (28.7 mo *vs* 19.1 mo) and progression-free survival (PFS, 26.4 mo *vs* 13.2 mo) were significantly longer in patients who received TS-1 adjuvant therapy compared to patients with gemcitabine-based adjuvant therapy. Patient selection bias could play a role in the excellent survival results because patients with larger than 4 cm pancreatic tumor were excluded.

Martin *et al*[48] analyzed data of 200 patients and so far this is the largest study on IRE of the pancreas. All patients received chemo- or chemoradiation therapy before the IRE (the chemotherapy was either gemcitabine- or FOLFIRINOX-based). In 50 patients IRE was used only for surgical margin extension and in 150 patients IRE was used without resection. In the resection + IRE group 75% of patients had body and neck tumors, while in the IRE group most patients (63%) had pancreatic head tumors. There was no significant difference in median OS between the two groups, however, the median OS was slightly longer in patients who received resection + IRE (28.3 mo *vs* 23.2 mo). The median OS and PFS for the entire group were 24.9 mo and 12.4 mo, respectively.

There were several studies which could not demonstrate survival benefit of IRE over standard of care chemotherapy with reported 9.3 mo median OS[51-54]. However, these are relatively early studies with potential biases (patient selection, different chemotherapeutic protocols, lack of experience) that may have influenced the outcomes. Although survival benefit of IRE was not proved compared to a matching control group, Lambert *et al*[54] recommended IRE in cases where unresectability was noted during laparotomy.

IRE can also be performed with laparoscopic approach using endoscopic or laparoscopic US guidance, but there are only 9 cases reported when laparoscopic technique was used[47,55,56]. In the study of Huang *et al*[47] 5 out of the 70 patients underwent laparoscopic ablation and their survival and complication results were not

Table 3 Studies evaluating intraoperative irreversible electroporation of pancreatic cancer

Author	Patients	Median OS(mo)	Guidancemodality	Major adverse events
Paiella *et al*[53], 2015	10	7.5	US	20%
Martin *et al*[48], 2015	200	24.9	US	22%
Kluger *et al*[52], 2015	50	7.7	Not reported	38%
Lambert *et al*[54], 2015	21	10.2	Not reported	24%
Yan *et al*[57], 2016	25	Not reported	US	16%
Vogel *et al*[50], 2017	15	16	US	53%
Huang *et al*[47], 2018	70	22.6	US	4%
Yang *et al*[105], 2020	74	53% (3 yr)[1]	US	12%
He *et al*[106], 2020	36	53.5% (2 yr)[2]	US	5%
He *et al*[49], 2020	167	16	US	N/A

[1]Survival probability at 3-year.
[2]Survival probability at 2-year. US: Ultrasound.

significantly different compared to the laparotomy group. Stillström *et al*[56] used an advanced stereotactic navigation system to perform laparoscopic IRE in 3 patients. The technical success rate was 100% and a prospective, randomized trial is planned. Tartaglia *et al*[55] reported a single case of laparoscopic IRE, where operators used laparoscopic ultrasound for guidance. The procedure was uneventful, and the 6-mo follow-up positron emission tomography-CT showed no fluorodeoxyglucose-uptake in the treated region.

In our experience probe placement-related bleeding is almost always seen due to the requirements of multiple probe placements and due to the rich blood supply of the upper gastrointestinal tract and porta hepatis. The IRE probe placements often performed *via* transgastric or transduodenal approach. During the open procedure the bleeding complications can be easily managed; in case of arterial bleed most of the times 5-10 min of manual compression is sufficient. Surgical vascular ligation is also readily available if needed. The other major advantage of open IRE over percutaneous IRE is the ability to sample and analyze suspicious lymph nodes with frozen section during the surgery and to detect obscure peritoneal metastases which are not seen on prior imaging. This reduces the chance to perform IRE on patients who have stage IV disease and unlikely would have benefit from the pancreatic ablation. Laparotomy is unavoidable anyway in a large number of LAPC patients since pancreatic head and neck tumors often cause gastric outlet obstruction. In these cases, IRE can be performed at the same time as the gastrojejunal bypass procedure.

Major adverse events of open IRE were reported in a wide range, from 4% to 53%. These can be laparotomy-related or IRE-related complications. Kluger *et al*[52] evaluated IRE-related and surgery related adverse events and found that 44% of grade 3-4 adverse events were IRE-related and 56% related to the surgical procedure. Authors concluded that IRE should not be considered as a minimally invasive treatment due to its high adverse event rate. Several authors did not describe whether they used imaging guidance during the IRE for probe placement and to monitor the ablation zone. Lack of imaging guidance may explain the high morbidity rates. Lambert *et al*[54] also included patients who underwent percutaneous IRE, but the percutaneous arm was closed after two patients due to high complication rate; one patient developed cholangitis, liver abscess, and biliary peritonitis resulting in surgical revision and antibiotic treatment, the other patient developed pancreatic fistula treated with stoma bag and antibiotics.

The most common IRE-related serious adverse events are pancreatic abscess formation, pancreatic fistula, gastrointestinal bleeding, and duodenal ulceration[47,48,57].

Percutaneous IRE is most commonly performed with CT guidance. CT-guided IRE requires administration of intravenous contrast to best visualize the tumor and its relationship to adjacent major vessels. US-guided percutaneous IRE has been reported [58-60] and US-guidance may be feasible in patients with low body mass index and without overlying gas-filled stomach and bowel loops, but in most patients

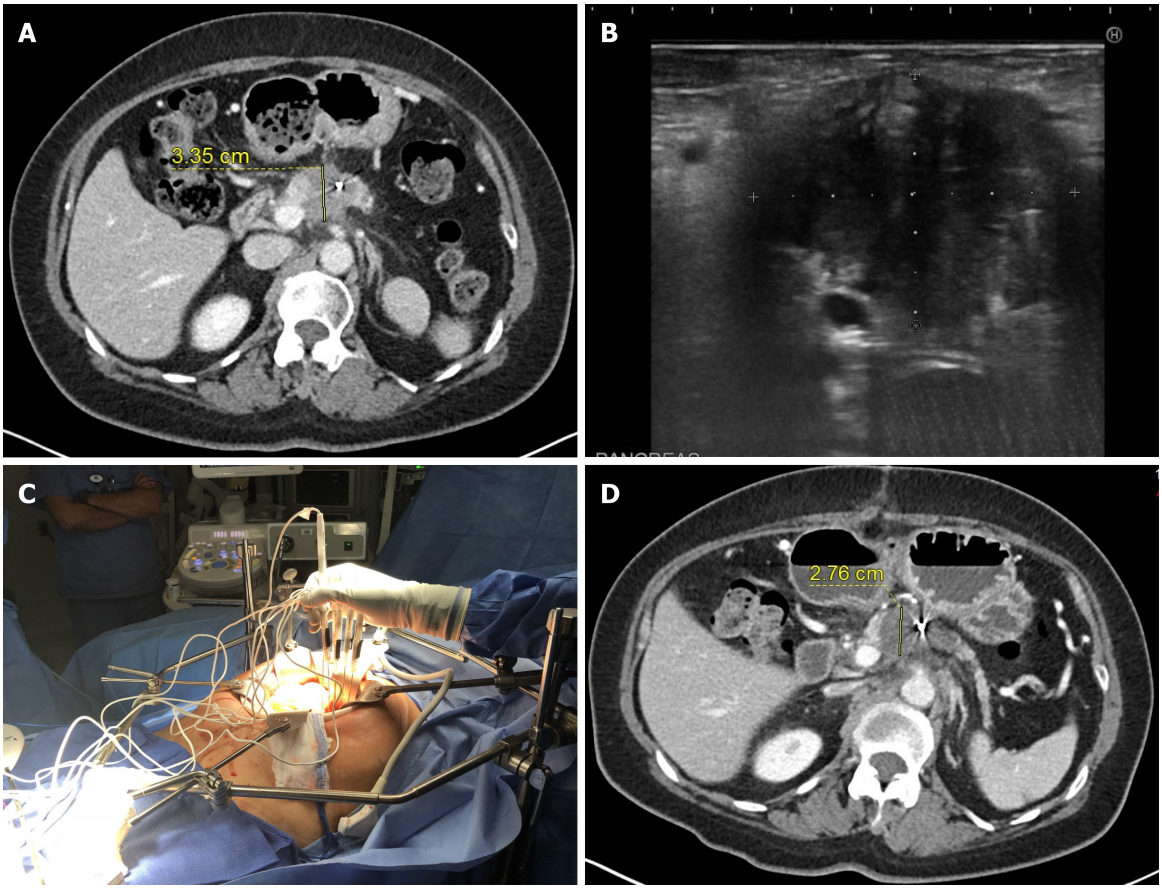


Figure 2 Intraoperative irreversible electroporation of a pancreatic adenocarcinoma. A: Pre-operative contrast-enhanced computed tomography image shows a hypodense mass in the head of the pancreas. A hyperdense fiducial marker, which was placed for prior radiation therapy, is also visible in the mass; B: Intraoperative ultrasound image shows 4 Í 3.2 cm hypoechoic mass in the pancreas; C: Intraoperative photo shows the patient with upper median laparotomy with ablation probes are placed directly into the pancreatic tumor, using ultrasound guidance; D: Follow-up imaging 1 mo after the irreversible electroporation (IRE) showed an avascular ablation zone covering the previously seen pancreatic tumor. The CA19-9 tumor marker decreased from 959 U/mL to 76 U/mL 1 mo after the ablation. The patient is still alive 31 mo after the IRE ablation.

percutaneous US is not suitable to guide pancreatic IRE ablation. The percutaneous approach is less invasive, and the hospital stay is shorter compared to open IRE, however, the management of procedural bleeding complications can be challenging and there are limitations to detect peritoneal and nodal metastases. The median OS of the percutaneous IRE studies is ranging from 10 to 19.8 mo. The longest overall survival was recently reported by Ma *et al*[51] The authors evaluated LAPC patients treated with gemcitabine plus IRE (33 patients) and gemcitabine alone (35 patients). The median OS of 19.8 mo in the combination therapy group indicates that including IRE into the treatment protocol provides significant survival benefit over gemcitabine alone (9.3 mo).

Narayanan *et al*[61] performed CT-guided percutaneous IRE in 50 LAPC patients. Nine of the 50 patients underwent a second IRE due to evidence of residual tumor. Three patients were downstaged and underwent surgical resection: pathologic examination showed negative margins in all the 3 patients with complete (1 patient) or partial (2 patients) tumor necrosis. Median OS was 14.2 mo. Most common major adverse event was abdominal pain in 7 patients. Ruarus *et al*[62] also published outcome of 50 patients who underwent CT-guided percutaneous IRE. This study included 16 patients who did not received neoadjuvant chemotherapy (FOLFIRINOX, gemcitabine, or capecitabine and oxaliplatin combination). For all patients, the median OS from IRE was 10 mo. Interestingly, the median OS was slightly longer in the patient group that received no chemotherapy, compared to those who did (11 mo *vs* 9 mo). Uni- and multivariate analysis revealed large tumor size, baseline CA19-9 Level higher than 2000 U/mL, and less than 50% reduction of CA19-9 at 3 mo follow-up as prognostic factors for poor survival.

A recent systematic review[63] compared open and percutaneous IRE. The mortality and morbidity rates were significantly higher in the open IRE group, meanwhile the median OS was found to be superior compared to the percutaneous approach. The

authors concluded that the treatment plan needs to be tailored individually.

There is a big difference between major centers in the rate of reported major adverse events ranging from 0% to 42%[58,62,64,65]. Belfiore *et al*[64] and Zhang *et al*[58] treated 20 and 21 patients, respectively, without any serious adverse events. On the other hand, most studies reported 20% or higher major adverse events after percutaneous IRE (Table 4).

Some patients may require special attention before IRE. Patients with pacemakers may not be a candidate for IRE. Metal stents and surgical clips may also contraindicate IRE. Månsson *et al*[66] reported a case of a patient with implanted metallic biliary stent who underwent IRE for the tumor in the head of the pancreas and developed severe late complications (persistent pain, abscess formation, diarrhea, peritonitis) within 8 wk of the procedure, that ended in emergency laparotomy and subsequent right hemicolectomy (for severe diarrhea). Martin *et al*[67] strongly recommends removal of metal biliary stents before IRE procedures. The metal stent can be removed endoscopically and replaced with a plastic stent or can be removed surgically with hepaticojejunostomy creation at the same laparotomy for open IRE. Dunki-Jacobs *et al*[68] demonstrated significant energy deflection during IRE in the presence of a metal stent which can lead to high current conditions, incomplete ablation, and possible thermal injury to adjacent organs. On the other hand, Melenhorst *et al*[69] successfully used IRE to treat a patient with hilar cholangiocarcinoma who had metallic stent in place. In our practice we haven't experience increased rate of complications of IRE in patients with metal stent in place (Kis *et al* unpublished data). At this point, the relationships between metal stent and IRE complications are not fully understood.

The non-thermal nature of IRE and its ability to preserve the structural integrity of blood vessels and bile ducts made IRE the preferred ablation modality in the pancreas. There are several ongoing clinical studies investigating the role of IRE in pancreatic cancer patients and to identify potential combination treatments which could improve survival (Table 5).

MINIMALLY INVASIVE TREATMENTS OF PANCREATIC CANCER LIVER METASTASES

At the time of diagnosis 53% of patients have distant metastases from pancreatic cancer and the 5-year survival of patients with metastatic disease is only 3%[1]. Liver is the most common site of metastasis with approximately 90% of patients develop hepatic metastasis[6]. In very selected cases of oligometastatic liver disease surgery can be considered[70-72], but the majority of patients are unresectable[73]. Chemotherapy is the only treatment option for most patients with metastatic pancreatic cancer[74]. The ACCORD11 trial reported 11.1 mo survival in metastatic pancreatic cancer patients treated with FOLFIRINOX, but the treatment had significant grade 3 and 4 toxicities including neutropenia (45.7%), thrombocytopenia (9.1%), diarrhea (12.7%), sensory neuropathies (9.0%), and fever (5.4%)[6]. For unresectable patients with liver-dominant metastatic disease liver-directed therapies may offer survival benefit and can provide chemotherapy holiday.

Percutaneous liver ablation

Percutaneous liver ablation is a widely used technique to treat primary and secondary malignancies in the liver[75]. The results of thermal ablations that covers the entire metastatic lesion is comparable to the results of surgical resection[76]. Studies evaluating percutaneous liver ablation in metastatic pancreatic cancer are summarized in Table 6.

Park *et al*[77] published a retrospective study of 34 patients who underwent liver RFA for metastatic pancreatic cancer. Median OS was 14 mo. Tumor size of less than 2 cm and well differentiated histology were predictive factors of longer survival. Second ablation due to new or recurrent metastasis was performed in 18 patients (58.1%), and in 16 of them the hepatic disease was controlled with the repeated ablation. Nine and one patient underwent a third and fourth ablation session, respectively.

Hua *et al*[78] performed a retrospective analysis of 102 pancreatic cancer patients who underwent RFA of liver metastases. Median OS was 11.4 mo. Univariate analysis showed that tumors in the head of the pancreas, tumor size between 3 and 5 cm and neutrophil-lymphocyte ratio > 2.5 were associated with worse prognosis. Recently Lee *et al*[79] published a retrospective study of 60 patients who underwent liver RFA for metachronous pancreatic cancer metastases and compared survival to a group of 66 patients who received systemic therapy. The median OS was significantly longer in the

Table 4 Studies evaluating percutaneous IRE in pancreatic cancer

Ref.	Patients	Median OS from IRE	Guidance	Major adverse events
Belfiore *et al*[64], 2015	20	13.95[1] mo	CT	0%
Narayanan *et al*[61], 2017	50	14.2 mo	CT	20%
Zhang *et al*[58], 2017	21	N/A	CT/US	0%
Scheffer *et al*[65], 2017	25	11 mo	CT	40%
Månsson *et al*[59], 2019	24	13.3 mo	US	25%
Flak *et al*[60], 2019	33	10.7 mo	US	20%
Ruarus *et al*[62], 2020	50	10 mo	CT	42%
Ma *et al*[51], 2020	33	19.8 mo	CT	9%

[1]Mean value. CT: Computed tomography; US: Ultrasound.

RFA group (12 mo) compared to the systemic therapy group (9.1 mo).

Although MWA and cryoablation is widely used in liver malignancies, there are no dedicated reports to date on pancreatic cancer metastases. Since MWA has several advantages over RFA it can be assumed that MWA is at least as effective as RFA ablating pancreatic cancer liver metastases. Bailey *et al*[80] published a study which included 20 metastatic pancreatic cancer patients who underwent any kind of liver directed treatment. In this study 10 patients had MWA of liver metastasis, but no survival or complication data was reported. During last 10 years we performed MWA of pancreatic liver metastasis in only 2 patients despite we are a high-volume ablation center and using almost exclusively MWA for liver metastases (Figure 3). Our practice reflects the recommendations of Ghidini *et al*[81] who reviewed the available data of surgical hepatic metastasectomy and ablative techniques for metastatic pancreatic cancer. They concluded that despite the advancements in both fields, neither surgery nor ablation improved overall survival significantly during the last decade. Therefore, metastasectomy and ablation of liver metastasis are not recommended routinely for stage IV pancreatic cancer patients.

Transarterial chemoembolization of pancreatic cancer liver metastases

All trans-arterial embolization therapies for liver tumors are predicated upon the fact that the liver has dual blood supply and while the normal liver receives approximately 80% of the blood from the portal vein and 20% from the hepatic artery, the blood supply of tumors are almost exclusively is from the hepatic artery. Therefore, by embolizing the tumor feeding hepatic arteries, tumor ischemia can be achieved with minimal hypoxic damage to the normal liver parenchyma.

During transarterial chemoembolization (TACE) the embolic agent is delivered simultaneously with a chemotherapeutic agent. Direct intra-arterial administration of chemotherapy increases concentration in the target area and decreases systemic toxicities of the drug. Embolization reduces intra-tumoral blood flow, therefore prolongs the dwell time of chemotherapy within the tumor and further decreases the systemic side effects of the drug.

TACE is a well-studied intraarterial therapy in the management of hepatocellular carcinoma and colon cancer, but there is limited data on TACE in metastatic pancreatic cancer (Table 7). In the largest series Vogl *et al*[82] analyzed the data of 112 patients with metastatic pancreatic cancer who underwent TACE using a combination of mitomycin C, cisplatin, and gemcitabine, followed by iodized oil and 50 μm embolization microspheres until stasis. Median OS was 19.2 mo with an impressive 5-year survival rate of 50%. Azizi *et al*[83] retrospectively analyzed the data of 32 patients who were treated with TACE, using the same technique as Vogl *et al*[82]. The median OS was 16 mo. Sun *et al*[84] reported a very promising 23 mo median OS of the 27 patients who underwent TACE for metastatic pancreatic cancer. The authors found that TACE prolongs survival and improves quality of life.

Transarterial radioembolization of pancreatic cancer liver metastases

During transarterial radioembolization (TARE) treatment Yttrium-90 (Y90) containing microspheres are delivered into the arteries feeding the liver metastases (Figure 4). Y90 is a pure β-emitting isotope with a physical half-life of 64.2 h[85]. It has a high energy

Table 5 Active pancreas irreversible electroporation clinical trials (source: ClinicalTrials.gov)

Title	Location	Hyperlink	Patients	Estimated completion
An open-label, multicenter, prospective study of IRE (Nano Knife) combined with radiotherapy and chemotherapy in patients with LAPC	Shanghai, China	https://clinicaltrials.gov/ct2/show/NCT04310553	40	December, 2020
Ablation of unresectable LAPC with IRE system	Teaneck, New Jersey, United States	https://clinicaltrials.gov/ct2/show/NCT03614910	30	May, 2023
Chemotherapy followed by irreversible electroporation in patients with unresectable LAPC	Aalborg, Denmark	https://clinicaltrials.gov/ct2/show/NCT04093141	30	May, 2024
Chemotherapy and IRE in the treatment of advanced pancreatic adenocarcinoma	Louisville, Kentucky, United States	https://clinicaltrials.gov/ct2/show/NCT03484299	20	December, 2023
PANFIRE-3 trial: Assessing safety and efficacy of IRE + Nivolumab + CpG for metastatic pancreatic cancer	Amsterdam, North-Holland, Netherlands	https://clinicaltrials.gov/ct2/show/NCT04612530	18	October, 2022
Outcomes of ablation of unresectable pancreatic cancer using the nanoknife IRE system	Baltimore, Maryland, United States	https://clinicaltrials.gov/ct2/show/NCT02041936	12	December, 2021
Immunotherapy and IRE in the treatment of advanced pancreatic adenocarcinoma	Louisville, Kentucky, United States	https://clinicaltrials.gov/ct2/show/NCT03080974	10	April, 2022
A study of the use of IRE in pancreatic ductal cancer	Toronto, Ontario, Canada	https://clinicaltrials.gov/ct2/show/NCT03257150	47	September, 2021
Safety and efficacy of IRE for LAPC	Seoul, Korea, Republic of	https://clinicaltrials.gov/ct2/show/NCT02898649	100	August, 2019
IRE (Nano Knife) for the treatment of pancreatic adenocarcinoma	Poitiers, France	https://clinicaltrials.gov/ct2/show/NCT03105921	20	June, 2020
IRE for inoperable hepatic and pancreatic malignancy	Hong Kong	https://clinicaltrials.gov/ct2/show/NCT02822716	35	December, 2021
Phase II/III of randomized controlled clinical research on IRE synchronous chemotherapy for LAPC	Guangzhou, Guangdong, China	https://clinicaltrials.gov/ct2/show/NCT03673137	120	November, 2021
Anti-tumor immunity induced by IRE of unresectable pancreatic cancer	Guangzhou, Guangdong, China	https://clinicaltrials.gov/ct2/show/NCT02343835	20	January, 2025
A pivotal study of safety and effectiveness of Nano Knife IRE for stage 3 pancreatic cancer	USA, Multicentre	https://clinicaltrials.gov/ct2/show/NCT03899636	528	December, 2023
Immunologic signatures following surgery for pancreatic cancer	Durham, North Carolina, United States	https://clinicaltrials.gov/ct2/show/NCT03001518	30	April, 2027

IRE: Irreversible electroporation; LAPC: Locally advanced pancreatic cancer.

radiation with average β-emission is 0.9367 MeV, with a mean tissue penetration of 2.5 mm and maximum tissue penetration of 11 mm[86,87], allowing delivery of high radiation doses to hepatic tumors with a "cross-fire" mechanism between the Y90 microspheres, while limiting the radiation dose to the surrounding liver parenchyma [88]. The antitumoral effect of Y90 is thought to be secondary to irreversible damage to tumor epithelial, stromal, and endothelial cells[89]. The absorbed dose of Y90 microspheres in the liver may be heterogeneous as it depends on hemodynamics and intratumoral vessel density[90]. The injected microspheres implant mostly in the terminal arterioles of tumors[88] in a 3: 1 to 20: 1 ratio compared to normal liver, with a preferential deposition in the tumor periphery[91]. There are currently two type of Food and Drug Administration-approved microspheres on the market: a glass (TeraSphere, Boston Scientific, Marlborogh, MA) and a resin (SIR-Spheres, Sirtex Medical Pty. Ltd, St Leonards, Australia) based microspheres. The two types of Y90 microspheres differ in several physical parameters. The most important difference is the approximately 50 times higher radioactivity/beads in glass microspheres compared to resin.

Table 6 Studies evaluating percutaneous liver ablation in metastatic pancreatic cancer

Ref.	Modality	Patients	Median OS from ablation	Guidance	Major adverse events
Park *et al*[77], 2012	RFA	34	14 mo	US	Not reported
Hua *et al*[78], 2017	RFA	102	11.4 mo	US	9.8%
Lee *et al*[79], 2020	RFA	94	12 mo	US (n = 91), CT (n = 3)	8.5%
Bailey *et al*[80], 2019	Mixed	20	9.7 mo	Mixed	N/A

US: Ultrasound; CT: Computed tomography.

Table 7 Transarterial chemoembolization in liver-metastatic pancreatic cancer

Ref.	Patients	Median OS from TACE	Technique
Vogl *et al*[82], 2015	112	19.2 mo	cTACE after mitomycin C, cisplatin, and gemcitabine chemoperfusion
Azizi *et al*[83], 2011	32	16 mo	cTACE after mitomycin C, cisplatin, and gemcitabine chemoperfusion
Sun *et al*[84], 2017	27	23 mo	Chemoperfusion with gemcitabine, oxaplatin, and irinotecan plus embolization with Lipiodol + pirarubicin or DEB with pirarubicin

OS: Overall survival; TACE: Transarterial chemoembolization; cTACE: Conventional transarterial chemoembolization.

TARE is generally better tolerated by patients than TACE. TARE was associated with better quality-of-life scores compared to TACE in patients with hepatocellular carcinoma[92]. Similarly, fewer side effects were reported after TARE compared to TACE in patients with intrahepatic cholangiocarcinoma and liver-dominant metastatic breast cancer patients[93-95].

Three workgroups published studies that evaluated the safety and efficacy of TARE in liver-dominant metastatic pancreatic cancer (Table 8)[96-100]. Michl *et al*[96] published a retrospective study of 19 patients treated with Y90-labelled resin microspheres. Six (31%) grade 3 or higher toxicity was reported. Overall survival was 9 mo. Kim *et al*[98] also used Y90-labelled resin microspheres in 33 patients and reported 8.1 mo median OS. Grade 3 adverse events were noted in 2 patients. Very recently we reported our experience of Y90 TARE using Y90-labelled glass microspheres in 26 patients with liver dominant metastatic pancreatic cancer[100]. Median OS was 7 mo and 3 patients (11.5%) had grade 3 clinical toxicity in this study. We found that longer hepatic progression free survival was associated with younger age (< 65 years) and decreased or stable CA19-9 tumor marker level following TARE treatment.

There was no head-to-head comparison between TACE and TARE, but it appears that TACE resulted in better OS in patients with metastatic pancreatic cancer. The reason for this difference is unknown.

CONCLUSION

The introduction of minimally invasive technologies in cancer care opened new therapeutic options for patients with malignancies beyond surgery, chemotherapy and radiation treatment. Interventional oncology is becoming the fourth pillar of cancer care besides the classic trio of medical oncology, surgery and radiation oncology. The data presented above demonstrates only modest improvement in overall survival of patients with LAPC and patients with metastatic pancreatic cancer, but still this is a significant step forward in the treatment of a disease which has dismal prognosis. In the current medical practice these new minimally invasive treatments are used mostly as salvage therapies, when a patient has no other option. This approach introduces a selection bias into the studies and may mask the real potential of these novel treatment modalities. Despite the selection bias, they have one obvious advantage, the low mortality and morbidity rates of image guided-procedures compared to surgery,

Table 8 Transarterial radioembolization in liver-metastatic pancreatic cancer

Ref.	Patients	Median OS from TARE	Technique
Michl *et al*[96], 2014	19	9 mo	Resin microspheres
Kim *et al*[97], 2016[1]	16	12.5 mo	Resin microspheres
Kim *et al*[99], 2017[1]	24	6 mo	Resin microspheres
Kim *et al*[98], 2019[1]	33	8.1 mo	Resin microspheres
Kayaleh *et al*[100], 2020	26	7 mo	Glass microspheres

[1]Patient cohort in studies by Kim *et al* may overleap. OS: Overall survival; TACE: Transarterial chemoembolization.

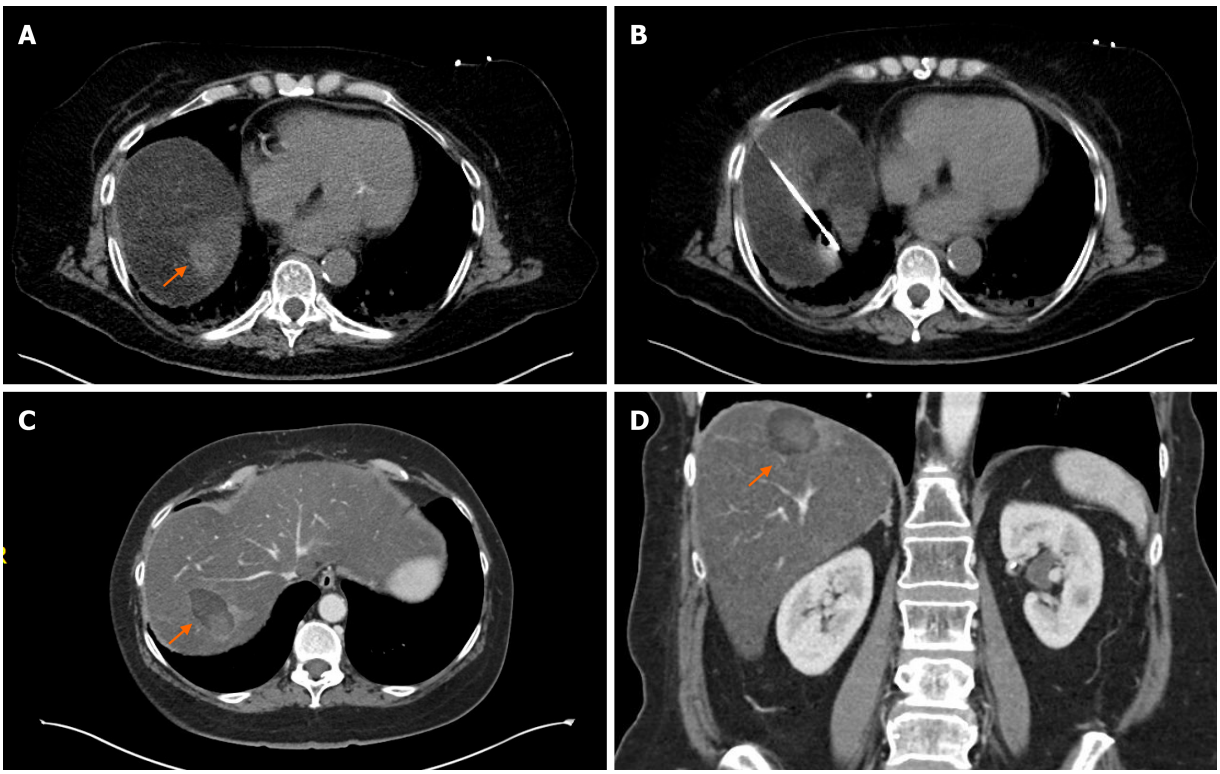


Figure 3 Percutaneous computed tomography-guided microwave ablation of a solitary pancreatic adenocarcinoma liver metastasis. A: 62-year-old female presented with a 2 cm solitary liver metastasis (orange arrow), 9 mo after the surgical resection of the primary tumor from the pancreatic head; B: Intraprocedural image shows the microwave antenna in the liver metastasis; C and D: Axial and coronal contrast-enhanced computed tomography images 1 mo after the ablation demonstrates a 4.3 Í 2.7 cm hypodense ablation zone (orange arrows). There was no evidence of residual or new metastases in the liver. Patient is currently on systemic therapy 2 mo after the ablation without evidence of disease.

radiation therapy and even to chemotherapy. Interventional oncology has many tools in its armamentarium to manage patients with primary and secondary pancreatic cancer; however, most of the new treatment options are in the experimental phase and only performed by large-volume centers.

We are in an era of a paradigm shift from conventional oncology to immuno-oncology which could promote the concurrent use of ablative technologies together with immune therapies[45,101-104]. It has been found that ablation of solid tumors triggers a tumor-specific immune response which can be beneficial in combination with immune checkpoint inhibitors or other immune modulator agents. Future studies are needed to define the specific role of interventional oncology in every stages of pancreatic cancer with special attention on the immunological effect of ablative techniques.

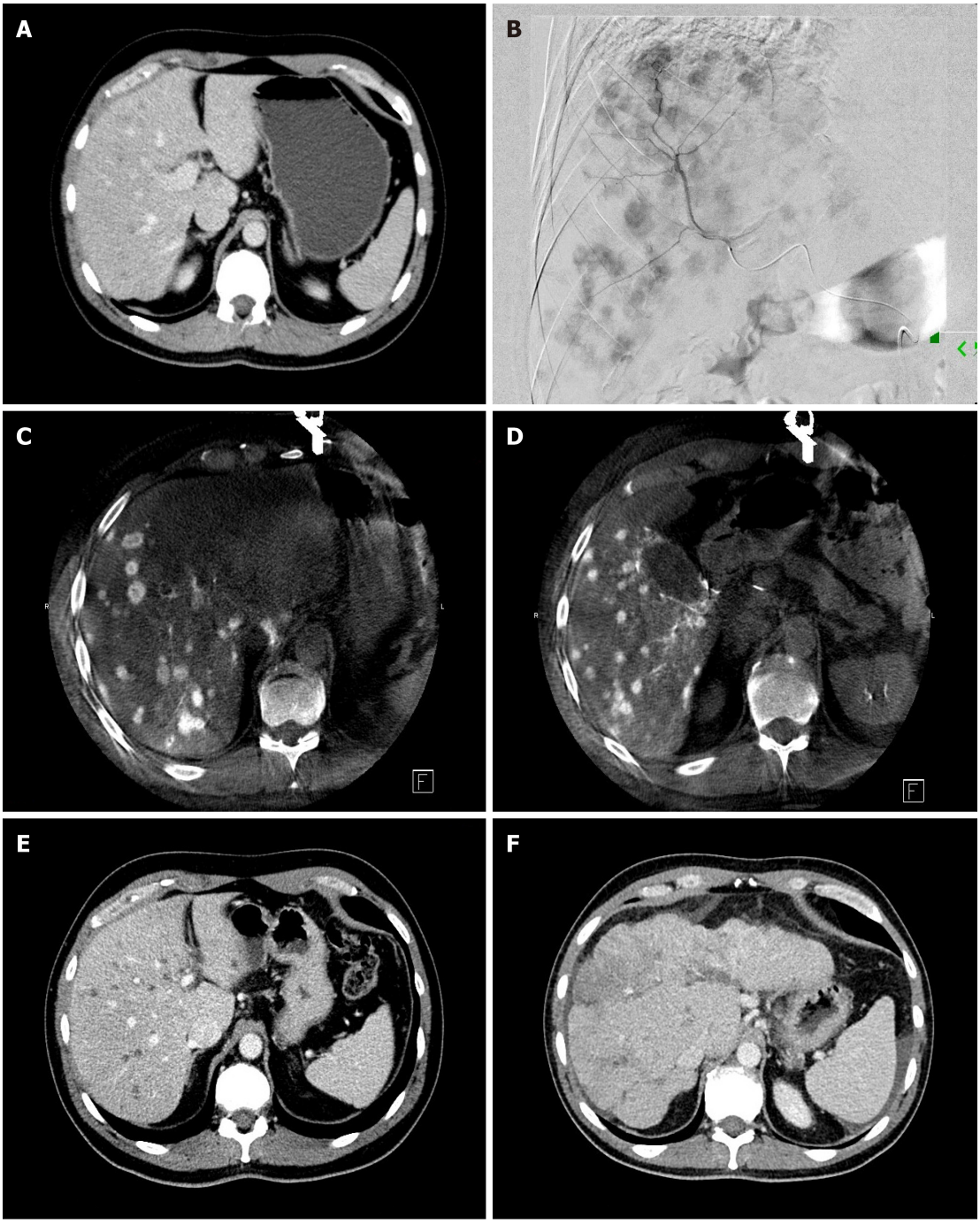


Figure 4 Transarterial radioembolization of pancreatic adenocarcinoma liver metastases. A: Pre-treatment contrast-enhanced computed tomography (CT) image of a 45-year-old man with pancreatic cancer demonstrates bilobar multifocal mildly hypodense liver metastases; B: Hepatic angiogram with contrast injection into the anterior division of the right hepatic artery shows multifocal arterially enhancing liver metastases; C and D: Intra-procedural cone-beam CT images of the liver during selective contrast injection into right hepatic artery shows multifocal arterially enhancing liver metastases; E: Contrast-enhanced CT image 1 mo after bilobar transarterial radioembolization (TARE) shows hypodense, "burnt out" liver metastases. The patient CA19-9 Level decreased from 882 U/mL pre-procedure to 56 U/mL. One year after the initial TARE new liver lesions appeared and his CA19-9 increased to 530 U/mL. He underwent a second round of bilobar TARE, and his CA19-9 Level decreased to 233 U/mL; F: Contrast-enhanced CT image 18 mo after the initial TARE shows shrunken liver with lobulated, nodular borders consistent with TARE-induced pseudocirrhosis. The patient died 22 mo after the initial TARE due to extrahepatic and hepatic progression of his disease.

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REVIEW

Comprehensive review of diagnostic modalities for early chronic pancreatitis

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Abstract

Chronic pancreatitis (CP) is a progressive condition caused by several factors and characterised by pancreatic fibrosis and dysfunction. However, CP is difficult to diagnose at an early stage. Various advanced methods including endoscopic ultrasound based elastography and confocal laser endomicroscopy have been used to diagnose early CP, although no unified diagnostic standards have been established. In the past, the diagnosis was mainly based on imaging, and no comprehensive evaluations were performed. This review describes and compares the advantages and limitations of the traditional and latest diagnostic modalities and suggests guidelines for the standardisation of the methods used to diagnose early CP.

Key Words: Chronic pancreatitis; Pancreatic fibrosis; Early diagnosis; Ultrasound endoscopy; Endosonography; Elastography; Confocal laser endomicroscopy

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Core Tip: Chronic pancreatitis (CP) is a progressive disease that is difficult to diagnose at an early stage. This review evaluates the characteristics, strengths, and limitations of modalities for the diagnosis of early CP. This paper will be of interest to the readership

because the information presented here highlights multiple novel strategies, such as elastography and confocal laser endomicroscopy, some of which require further research and development to improve the diagnostic efficiency. This review will be highly beneficial for researchers and clinicians focusing on the management of this condition.

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INTRODUCTION

Chronic pancreatitis (CP) is a multifactorial inflammatory syndrome characterised by recurring epigastric pain and progressive fibrosis that eventually leads to pancreatic atrophy and calcification, ductal distortion, and exocrine and endocrine dysfunction[1,2]. In early pancreatitis, clinical features of the disease are mild and non-specific[3,4]. The global incidence of CP is 10 per 100000 persons per year, and significantly more men than women are affected. In Japan, the overall prevalence of early CP is 4.2 per 100000 persons, and the annual incidence is 1.0 per 100000 persons[5-7]. Several classifications and diagnostic recommendations for CP have recently been proposed[3,4,8-10]. For advanced or end-stage CP, morphologic changes characteristic of fibrosis, duct dilation, calcifications, or atrophy can be detected with imaging modalities and functional failures can be evaluated through functional tests. However, there is no universally accepted diagnostic method for CP[4]. Furthermore, due to a lack of specific clinical presentation or morphologic features, diagnosing CP at an early stage is challenging[11]. Thus, it is critical to combine what is known about the aetiology, risk factors, clinical symptoms, imaging features, and pancreatic function test results to paint a clinical picture[12]. In this review, we compare different diagnostic modalities including several recently developed testing approaches such as endoscopic ultrasound (EUS)-elastography and EUS-guided needle-based confocal laser endomicroscopy (nCLE). We also suggest the development of standardised guidelines for the early diagnosis of CP, which will resolve the challenge of diagnosing early CP.

AETIOLOGY AND CLINICAL PRESENTATION

CP is a fibro-inflammatory disease with various aetiologies that is typically diagnosed using the TIGAR-O criteria[13] (Table 1) and M-ANNHEIM classification system[9]. These international classification systems incorporate common aetiological risks including alcohol and nicotine consumption, genetic mutations and polymorphisms, metabolic disorders, ductal obstruction, immunological factors, and idiopathic pancreatitis[14-16]. Among these factors, alcohol abuse is the most common aetiology of CP and is diagnosed in 42%-77% of patients with CP[17,18]. A prospective study reported that lifestyle-related factors such as alcoholism or smoking are closely associated with the occurrence and progression of CP[19,20]. Ethanol and nicotine are associated with oxidative stress, which activates quiescent pancreatic stellate cells that play an essential role in the inflammatory response and pathological progression of CP, eventually leading to irreversible pancreatic parenchyma damage and fibrosis[21-25]. Chronic alcohol consumption is also thought to increase gut permeability and decrease the phagocytic capacity of Kupffer cells, rendering them unable to detoxify circulating endotoxins; thus, heavy drinkers (> 80 g alcohol/d or more than 5 drinks/d) are susceptible to pancreatic diseases[26,27]. The revised TIGAR-O criteria[13] are used as a grading system for alcohol and nicotine consumption and are preferred to the exposure assessment (Table 1). Furthermore, genetic mutations are usually associated with CP onset, and different genotypes may lead to different effects and disease outcomes. A large Chinese cohort study focusing on four CP-associated genes (*SPINK1*, *PRSS1*, *CTRC*, and *CFTR*) found that patients with gene mutations had earlier disease onset than patients without. The study also found that genetic mu-

of interest for this manuscript.

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Table 1 Revised TIGAR-O aetiology criteria

Toxic-metabolic
Alcohol-related
0 to < 1 drinks/d
1-2 drinks/d
3-4 drinks/d
5 or more drinks/d
Smoking
Non-smoker (< 100 cigarettes in lifetime)
Past smoker
Current smoker (patients undergoing both past and ongoing cigarette exposure)
Other, not otherwise specified
Hypercalcaemia (total calcium levels > 12.0 mg/dL or 3 mmol/L)
Hypertriglyceridemia
Hypertriglyceridemia risk (fasting glucose > 300 mg/dL; non-fasting glucose > 500 mg/dL)
Hypertriglyceridemia in acute pancreatitis (history of glucose > 500 mg/dL in first 72 h of AP onset)
Medications
Toxins, other
Chronic kidney disease [CKD Stage 5: end-stage renal disease (ESRD)]
Other, not otherwise specified
Idiopathic
Early-onset (< 35 yr of age)
Late-onset (> 35 yr of age)
Genetic
Suspected; no or limited genotyping available
Autosomal dominant (Mendelian inheritance-single gene syndrome)
PRSS1 mutations (hereditary pancreatitis)
Autosomal recessive (Mendelian inheritance-single gene syndrome)
CFTR, 2 severe variants in trans (cystic fibrosis)
CFTR, < 2 severe variants in trans (CFTR-RD)
SPINK1, 2 pathogenic variants in trans (SPINK1-associated familial pancreatitis)
Complex genetics (non-Mendelian, complex genotypes +/- environment)
Modifier Genes (pathogenic genetic variants)
PRSS1-PRSS1 locus
CLDN2 locus
Others
Hypertriglyceridemia
Other, not otherwise specified
Autoimmune pancreatitis (AIP)/ steroid responsive pancreatitis
AIP Type 1—IgG4-related disease
AIP Type 2
Recurrent acute pancreatitis (RAP) and severe acute pancreatitis (SAP)
Acute pancreatitis (single episode, including date of event if available)

AP aetiology—Extra-pancreatic (excluding alcoholic, HTG, hypercalcaemia, genetic)
Biliary pancreatitis
Post-ERCP
Traumatic
Undetermined or not otherwise specified
Recurrent acute pancreatitis (number of episodes, frequency, and dates of events if available)
Obstructive
Pancreas divisum
Ampullary stenosis
Main duct pancreatic stones
Widespread pancreatic calcifications
Main pancreatic duct strictures
Localized mass causing duct obstruction

From the short form of TIGAR-O version 2 risk/aetiology checklist (2019)[13]. AP: Acute pancreatitis; CKD: Chronic kidney disease; HTG: Hypertriglyceridemia; ERCP: Endoscopic retrograde cholangiopancreatography.

tations were most common in patients with idiopathic chronic pancreatitis (ICP) than in patients with alcoholic chronic pancreatitis (ACP) or smoking-associated chronic pancreatitis (SCP)[28]. Patients with inherited *PRSS1* mutations have reportedly presented with specific imaging features including pancreatic atrophy, calcification, and main pan-creatic duct (MPD) dilatation, which have been termed the PRSS1 imaging triad[29]. Patients with *CFTR* gene mutations have presented with pancreatic atrophy.

An early diagnosis of CP is challenging due to nonspecific clinical presentation. Recurrent abdominal pain with radiation to the back is the most common symptom, with Wilcox *et al*[30] reporting that constant, mild pain with intermittent episodes of severe pain is described by 45% of patients. A history of acute pancreatitis (AP), especially recurrent AP, is a significant risk factor for early CP[4]. However, in some patients, CP is asymptomatic during the early stage, and steatorrhea or diabetes secondary to exocrine/endocrine dysfunction may be the first clinical manifestation of CP without pain[31]. If patients with CP are not diagnosed at an early stage, they have a higher risk of progressing to advanced or end-stage CP, which is characterised by multiple complications including severe pain, pancreatic insufficiency (endocrine or exocrine), metabolic bone disease, and pancreatic ductal adenocarcinoma (PDAC)[19,32,33]. It is currently accepted that an early or suggestive CP diagnosis can be made when three or more of the following clinical features are present: Abnormal serum or urine pancreatic enzyme concentrations; recurring upper abdominal pain; continuous heavy alcohol consumption (> 80 g alcohol/d); family history of hereditary CP or known sporadic high-risk mutations; and abnormal exocrine function (Table 2)[1,4,33,34]. Genetically mediated pancreatitis is likely in a young patient with clinical features of CP but without a history of risk factors such as smoking or alcohol consumption. However, early CP cannot be diagnosed using clinical manifestations alone.

IMAGING MODALITIES

The progression from normal pancreatic status to severe inflammation is typically subtle during which the patient experiences few specific symptoms. Thus, the majority of CP diagnoses are based on imaging features[1,4]. Imaging and radiographical evaluations of a patient with suspected CP should progress from a non-invasive approach to an invasive approach.

Ultrasound and computed tomography

Ultrasound (US) and computed tomography (CT) are recommended by various guidelines as the first-line non-invasive imaging approaches for evaluating patients with suspected CP[10,12]. Intraductal pancreatic calcification and parenchymal atrophy are

Table 2 Comparisons of diagnostic modalities

Modality	Diagnostic standards	Sensitivity	Specificity
Aetiology	TIGAR-O classification (version 2)[13].	-	-
Clinical presentation	Three or more of the following features: Abnormal serum or urine pancreatic enzyme concentrations; continuous heavy alcohol consumption (> 80 g alcohol/day or more than 5 drinks/day), family history of hereditary chronic pancreatitis, or known sporadic high-risk mutations; recurring epigastric abdominal pain; and abnormal exocrine function. Genetic pancreatitis should be suspected in young patients with clinical presentations but without a history of risk factors.	-	-
TA-US	Irregular main pancreatic duct with a diameter > 3 mm, hyperechoic pancreatic duct wall, or lobularity with stranding.	69% (95%CI: 54-80)	94%(95%CI: 90-100)
CT	Two or more of the following features: MPD within 2-4 mm; mild organ enlargement; irregular main pancreatic duct with ≥ 3 pathological side branches; pseudocysts ≤ 10 mm; and heterogeneous parenchyma.	75% (95%CI: 66-83)	91%(95%CI: 81-96)
MRI/MRCP	Two or more of the following features: MPD 2-4 mm; mild organ enlargement; irregular main pancreatic duct with ≥ 3 pathological side branches; pseudocysts ≤ 10 mm; and heterogeneous parenchyma.	Single-parametric: 77%; Multi-parametric: 91%	Single-parametric: 83%; Multi-parametric: 86%
ERCP	More than three pathological side branches plus a normal MPD.	82% (95% CI: 76-87)	94% (95% CI: 87-98)
EUS	More than two of the following seven criteria, including at least one of criteria 1-4: (1) Stranding; (2) Hyperechoic foci without shadowing; (3) Lobularity with honeycombing; (4) Lobularity without honeycombing; (5) Cysts; (6) Dilated side branches; (7) Hyperechoic main pancreatic duct margin.	61% (non-fibrosis); 84% (for fibrosis)	75% (non-fibrosis); 100% (for fibrosis)
EUS-EG	A strain ratio of > 10 or a mean strain histogram value of < 50 was associated with malignancy.The mean value can be used to diagnose mild or higher-grade fibrosis.	76.4%	91.7%
FE-1	Moderate EPI can be diagnosed based on an abnormal FE-1 level of < 200 μg/g, which has a high false-positive rate.	76.5%; 45.0% (mild ductal changes and insufficiency)	86.0%
ePFT	Peak bicarbonate concentration of < 80 mmol/L is considered abnormal and correlated with early fibrosis.	86% (95%CI: 67-100)	67% (95%CI: 13-100)
FNA	Ruling out malignancy and staging of CP. CEA testing: Cut-off value of 192 ng/mL. Molecular analysis: *KRAS* and *GNAS* mutations.	85% (pancreatic cancer)	98% (pancreatic cancer)
nCLE	A complementary modality for detecting subtle changes in early CP and helpful for distinguishing malignancies.	94.3% (cystic lesions); 90.3% (PDAC)	98.1% (cystic lesions); 89.5% (PDAC)

Sen: Sensitivity; Spec: Specificity; TA-US: Transabdominal ultrasound; MPD: Main pancreatic duct; CT: Computed tomography; MRI: Magnetic resonance imaging; EUS: Endoscopic ultrasound; EUS-EG: Endoscopic ultrasound elastography; FE-1: Faecal elastase-1; ePFT: Endoscopic pancreatic function test; nCLE: Endoscopic pancreatic function test; EPI: Exocrine pancreatic insufficiency; CP: Chronic pancreatitis; CEA: Carcinoembryonic antigen; MRCP: Magnetic resonance cholangiopancreatography; ERCP: Endoscopic retrograde cholangiopancreatography; FNA: Fine-needle aspiration; PDAC: Pancreatic ductal adenocarcinoma; AIP: Autoimmune pancreatitis; AP: acute pancreatitis; RAP: Recurrent acute pancreatitis; CKD: Chronic kidney disease; NCCP: Non-calcific chronic pancreatitis; ROC: Receiver operating characteristic; ROI: Region of interest; SD: Standard deviation; SR: Strain ratio.

considered the most specific and reliable sonographic signs of CP. However, the value of transabdominal US (TA-US) may be limited by the retroperitoneal location of the pancreas, which increases the sound wave distance to the pancreas, and by several other factors including bowel gas, obesity, and individual variations. Nevertheless, advances in US technology have significantly improved the diagnostic value of the modern abdominal US. A prospective observational cohort study reported a sensitivity of 0.69 (95%CI: 0.54-0.80) and specificity of 0.97 (95%CI: 0.90-1.0) for the detection of CP using TA-US[35]. The following US signs have been proposed for the diagnosis of early or suspected CP: An irregular main pancreatic duct > 3 mm; an hyperechoic pancreatic duct wall; or lobularity with stranding (Table 2)[1]. In addition also criteria of size, shape and mobility should also be taken into account[36,37].

CT is believed to be the best initial imaging modality for the diagnosis of CP, and some guidelines have suggested that all patients with suspected CP should undergo a baseline CT scan[10,12,38]. Ductal changes such as dilation, strictures, and contour irregularity and parenchymal or intraductal calcification, which have been deemed to constitute an independent pathophysiological process involved in the development of CP, can be identified on CT. Furthermore, baseline CT can be useful to rule out other intra-abdominal diseases, including pancreatic and upper gastrointestinal cancers,

which may present with similar symptoms of epigastric pain, weight loss, and maldigestion[38]. In addition, CT can also be used to monitor the progression of pancreatitis and its subsequent complications, such as pseudocysts and biliary obstruction. A systematic meta-analysis reported a sensitivity of 75% (95%CI: 66-83) and specificity of 91% (95%CI: 81-96) for the detection of CP using CT[39]. However, the value of CT is limited for patients with early CP as the parenchymal and ductal changes are subtle. Furthermore, both US and CT have high rates of false negatives due to their limitations. It has been recommended that the quantity, size, and location of pancreatic calcification need to be demonstrated for accurate and comprehensive fibrosis assessments[40,41]. A dilated main pancreatic duct (2-4 mm), mild organ enlargement, pseudocysts, and pathological side branches are considered to be the diagnostic signs of early CP on CT (Table 2)[1,4,33,34].

Magnetic resonance imaging

Patients with suspected pancreatitis should also undergo magnetic resonance imaging (MRI) prior to other evaluations using invasive investigations to rule out carcinoma [10]. MRI and MR cholangiopancreatography (MRCP) are recommended, especially in patients without specific changes detected on CT. MRI and MRCP are superior to CT for the identification of mild CP. For example, morphological changes such as duct dilatation and strictures and pathological side branches that are typical early CP signs are more easily detected on MRI than on CT. Furthermore, new MRI techniques have been applied to quantitatively evaluate the severity of pancreatic fibrosis and pancreatic exocrine dysfunction, including diffusion-weighted imaging (DWI), MR elastography, and T1-mapping of the pancreatic parenchyma[38,41-43]. A retrospective analysis conducted by Tirkes *et al*[44] proposed that T1-weighted MR signaling in the pancreas had a high sensitivity (77%, $P < 0.0001$) and specificity (83%, $P < 0.0001$) for detecting parenchymal abnormalities related to exocrine dysfunction and could be helpful for the assessment of suspected early CP. Another study showed that multiparametric mapping MRI (a combination of T1, T2, and apparent diffusion coefficient values) yielded a higher accuracy for the detection of CP than any sole component (sensitivity: 91.54%; specificity: 85.81%; $P < 0.001$)[45]. If a patient has a high probability of having CP but shows negative results on MRI or MRCP, secretin-stimulating MRCP (part of the exocrine function test) should be performed[38]. However, MRCP does not always detect subtle pancreatic changes, especially when compared to endoscopic retrograde cholangiopancreatography (ERCP), which can be used to diagnose early CP based on the criteria of more than three pathological side branches and a normal main pancreatic duct (MPD) with a specificity of 94%. However, ERCP is not readily available everywhere, and it is operator-dependent and challenging to conduct[46]. The revised Cambridge classification system[47] recommends the following MRCP criteria for the diagnosis and evaluation of early CP: MPD of 2-4 mm; pseudocysts ≤ 10 mm; and irregular MPD with ≥ 3 pathological side branches (Table 2)[1,35].

Endoscopic ultrasound

Endoscopic ultrasound (EUS) has been reported to be the most sensitive modality for detecting fibrosis in patients with CP[4]. EUS is also superior to non-invasive imaging tools in diagnosing parenchymal and ductal changes, especially during the early stage of the disease. Therefore, when CT and MRI show negative results in patients who are suspected of having CP, EUS should be performed[38]. The majority of the EUS diagnostic criteria are based on the Rosemont classification system published in 2009 [48], which attempted to define each EUS criterion precisely to achieve excellent interobserver agreement. Based on the Rosemont classification system, EUS can be used to detect subtle changes in the structure of the pancreatic parenchyma and ducts even before traditional imaging and functional testing. EUS can also be used to treat pancreatitis-related symptoms or complications such as peripancreatic fluid and pseudocyst drainage[49,50]. Standard EUS findings include changes in the parenchyma (hyperechoic foci with or without shadowing, lobularity with or without honeycombing, pseudocysts, and hyperechoic stranding) and ducts (MPD irregularity, dilated side branches, hyperechoic main pancreatic duct wall, MPD dilation (3 mm at the head, 2 mm in the body, or 1 mm at the tail], and MPD stones)[48]. When more than two of the following pancreatic features are found on EUS, including at least one of the first three criteria, the patient is diagnosed with early CP: Lobularity with or without honeycombing, hyperechoic foci without shadowing, stranding, cysts, dilated side branches, and hyperechoic main pancreatic duct margins (Table 2)[1,34].

EUS findings such as hyperechoic foci and strands, parenchymal lobularity, and a hyperechoic ductal wall are considered to be signs of pancreatic fibrosis[51]. A study conducted in 2016 using a quantitative receiver operating characteristic (ROC) curve analysis to evaluate the accuracy of EUS features revealed that four or more standard EUS features achieved the best balance of sensitivity (61%; 95%CI: 46.8-73.5) and specificity (75%; 95%CI: 42.8-94.2) (AUC = 0.68) for predicting abnormal pancreatic histopathology alterations of non-calcific pancreatitis. Nevertheless, the Spearman rank correlation coefficient (*r*) calculated in the study demonstrated a poor correlation between standard EUS features and histopathology ($r = 0.24$, $P < 0.05$), suggesting that EUS is not an independent diagnostic modality for early CP, and that other influencing factors, such as age, sex, BMI, and environmental exposure, should also be included in the diagnostic algorithm[52]. However, a different retrospective study reported that the sensitivity (84%; 95%CI: 69-100) and specificity (100%; 95%CI: 40-100) of the detection of fibrosis using EUS becomes superior as the disease progresses[53,54]. A significant advantage of EUS is its ability to image the side branches and mild contortions of the MPD in normal individuals, especially in elderly individuals. Side branches exceeding 1 mm are considered abnormal. However, by the same token, there is potential for over-diagnosis of early CP especially in elderly individuals due to the very high sensitivity. Therefore, there is potential of over-diagnosis of early CP (Figure 1) by EUS and it should be performed and interpreted in the right clinical setting. Contrast-enhanced EUS with secretin stimulation and quantitative elastography are needed to improve the diagnostic accuracy for mild or suspected CP[55].

Elastography

Fibrosis in CP generally results in increased stiffness of the pancreatic parenchyma or ducts, which can be qualitatively or quantitatively evaluated based on strain or shear wave speed using elastography[56,57]. Together, EUS and elastography have been used to quantitatively measure the severity of pathological changes in CP, including parenchymal fibrosis[58]. EUS elastography (EUS-EG) involves the compression of a target tissue with an echo-endoscopic probe. The resulting strain on the tissue depends on its hardness or softness and can be compared with that of normal surrounding tissue using a three-colour system: Red, soft tissue; green, average hardness; and blue, hard tissue[59]. The quantitative analysis is based on a strain ratio (SR) calculation. Tissue stiffness is measured in a target lesion (region of interest ROI A) and a normal reference area (ROI B), which can be located either in the surrounding normal parenchyma or the wall of the gastrointestinal tract, and the SR is calculated as the quotient A/B[60]. Specific software is used to differentiate between lesions and normal tissues based on the negative correlation between SR and tissue elasticity. Strain histogram analysis is another quantitative elastography method, involving the calculation of the average hue histogram value over several compression cycles. The mean value of the strain histogram reflects the global stiffness or elasticity of a focal lesion based on the selected ROI calculation. Standard deviation (SD) and other parameters can also be used to further describe the hue distribution within the ROI [61]. Previous studies have reported that the evaluation based on the SR and histogram obtained by quantitative EUS-EG can be used as a supplementary approach for assessing the severity of parenchymal fibrosis and making a differential diagnosis of pancreatic masses[2,4,56,57,62]. A prospective study revealed a strong linear correlation between the number of EUS criteria for CP (according to the Rosemont classification system) and the SR ($r = 0.813$; $P < 0.0001$). Additionally, the area under the ROC curve was 0.949 (95%CI: 0.916-0.982), indicating outstanding diagnostic accuracy (91.1%, $P < 0.0001$) based on a cut-off SR of 2.25[63]. Itoh *et al*[64] used quantitative EUS-EG to diagnose the grade of pancreatic fibrosis and proposed that the pancreatic fibrosis grade is significantly correlated with histogram parameters, especially the mean histogram value ($r = -0.75$). When the mean value was used to diagnose mild or higher-grade fibrosis, the area under the ROC curve exceeded 0.9 (95%CI: 0.82-0.98) with a sensitivity of 76.4% and specificity of 91.7% (Table 2). An SR > 10 or a mean strain histogram value < 50 has been associated with malignancy, suggesting that EUS elastography is a useful supplementary modality to rule out malignant lesions of the pancreas due to its high negative predictive value[65]. A correlation between elasticity and EUS criteria has also been reported when elastography evaluations are based on the shear wave, which enriches the elastography approach for CP diagnosis[66-69]. As elastography is able to efficiently and accurately assess pancreatic fibrosis, combining EUS and elastography can greatly enhance the accuracy of detecting subtle or mild changes in patients with early or higher-grade CP.

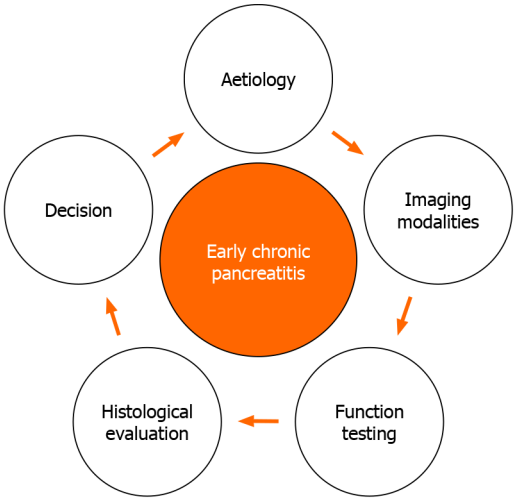


Figure 1 Outline of comprehensive diagnosis of early chronic pancreatitis.

PANCREATIC FUNCTION TESTING

Exocrine pancreatic insufficiency (EPI) is a common complication of CP mainly caused by the impairment of the production and secretion of enzymes in the pancreas. Symptoms of EPI include maldigestion of nutrients, unexpected weight loss, and steatorrhea due to fat maldigestion[32]. Pancreatic function testing (PFT) is recommended for the diagnosis of CP, especially in patients with non-specific and inconclusive morphological or imaging features of the disease[33,38]. PFT includes direct tests, which collect and analyse pancreatic secretions after a hormone stimulus, and indirect tests, which assess pancreatic function *via* faecal elastase-1(FE-1) or a breath test.

Direct PFT is conducted by determining the pancreas enzyme output after stimulation with cholecystokinin (CCK). The combination of secretin-stimulated MRI (s-MRI) and EUS-based PFT allows for the assessment of the bicarbonate concentration in the pancreatic juices as well as an improved morphological analysis[70]. S-MRI is considered to be a safe and non-invasive technique that enhances the visualisation of the ductal system by stimulating the bicarbonate-rich fluid filling the MPD and its side branches. EPI is diagnosed when the bicarbonate concentration is < 80 mmol/L after secretin stimulation[71]. Following intravenous secretin (0.2-0.3 μg/kg within 1-2 min) administration, an increase in hyperintense fluid content is observed in the small intestine 10-13 min thereafter. During the post-secretin stimulation stage, the MPD has a larger diameter and can be more easily evaluated, and pancreatic ductal compliance, which is associated with ductal stiffness or calcification, can be assessed. Healthy, nonfibrotic pancreatic parenchyma and ducts without strictures or calcification have an elastic capacity for accommodating pancreatic juice fluid without dilatation, and the diameter will not increase by more than 1 mm from the baseline value after secretin stimulation[10,23,72]. Exocrine pancreatic function can be quantitatively and semi-quantitatively evaluated by assessing the production and excretion of bicarbonate from pancreatic glands[10,73]. Thus, s-MRI can be applied to diagnose early or mild CP based on its superior ability to detect early pathological changes, such as the presence of side branches and an enlarged or restricted MPD, compared to CT or standard MRI. Several studies have reported significant differences between patients with a normal pancreas and early pancreatitis using s-MRI and correlations between s-MRI findings and the histopathological features of CP[74-76]. Similarly, EUS can be used in the endoscopic pancreatic function test (ePFT), which has been reported to have a sensitivity of 86% (95%CI: 67-100) and specificity of 67% (95%CI: 13-100) for the diagnosis of early fibrosis[53]. A retrospective study reported that a peak bicarbonate concentration of < 80 mmol/L on ePFT is considered abnormal and proposed a correlation between the peak bicarbonate concentration and fibrosis score (r = -0.57; P = 0.016). However, other studies have also claimed that the specificity and concordance for CP diagnosis were more accurate in a shortened ePFT with the use of a lower cut-off value; more prospective studies are required for further validation of the ePFT (Table 2)[77,78].

Although direct PFT is generally considered to be the gold standard for EPI, indirect PFT is useful for screening patients with risk factors for CP, as it is non-invasive and convenient. Indirect PFT can be performed using faecal chymotrypsin or FE-1 assays, 72-h faecal fat measurement, bentiromide (NBT-PABA) tests, and fluorescein dilaurate tests. FE-1 assessment is the most commonly used PFT method as it is stable throughout the intestinal tract. Furthermore, EPI can be diagnosed based on an abnormal FE-1 level (< 200 μg/g), though this diagnostic method is limited by a high false-positive rate (Table 2)[79]. FE-1 has also been reported to correlate with pancreatic duct changes, with a sensitivity of 76.5% and a specificity of 86% ($P < 0.05$) for moderate to severe changes at a cut-off of 200 μg/g. However, the correlation of FE-1 with mild ductal changes and insufficiency was not as strong, with a sensitivity of 45% [80,81].Therefore, the FE-1 test is not an accurate method for the functional diagnosis of EPI and should not be used to diagnose early CP.

HISTOLOGICAL EVALUATION

EUS-guided tissue acquisition

While the histopathological examination is considered the gold standard of disease diagnosis, direct acquisition of pancreatic tissue is limited due to the retroperitoneal location of the organ. Tissue acquisition with EUS (EUS-TA) can be used to obtain pancreatic tissue samples for microscopic analysis and establish a cytopathological diagnosis of the gastrointestinal tract or adjacent lesions[82]. In patients with suspected pseudo tumoural masses or cystic lesions in the setting of CP, a histopathological examination is necessary to eliminate malignant lesions from the differential diagnosis[72,82]. EUS-TA can be achieved *via* EUS fine-needle aspiration (EUS-FNA) and EUS-fine needle biopsy (EUS-FNB), which is the current gold standard for tissue acquisition from solid masses. FNA is used to rule out malignancy from the differential or to stage CP and can provide both cytological and histological evaluations. FNA is reported to be superior to other modalities for the evaluation of cystic pancreatic lesions in patients with concurrent CP[83]. The fluid obtained by FNA can be tested for amylase and carcinoembryonic antigen (CEA). Moreover, the CEA level can be used to distinguish mucinous and non-mucinous lesions with a cut-off value of 192 ng/mL[83,84]. Additionally, the molecular analysis of mutations in the *KRAS* and *GNAS* genes using the FNA specimen can distinguish malignant lesions from benign cystic lesions[85,86]. FNA has also been reported to be useful for evaluating solid lesions and suitable for distinguishing autoimmune pancreatitis (AIP) from pancreatic cancer[87,88]. FNA has been reported to have a sensitivity of 85% and specificity of 98% for the diagnosis of pancreatic cancer (Table 2)[89]. However, according to a current retrospective analysis of multicentric databases, FNB showed a higher diagnostic sensitivity than FNA for distinguishing between inflammatory masses and malignant lesions in the setting of CP[90]. However, histopathological evaluation by FNB may cause needle tract seeding, which should be taken into consideration when conducting FNB on patients with resectable solid masses[62]. Thus, the appropriate method of tissue acquisition depends on the specific type of pancreatic lesion and the purpose of diagnosis. EUS-TA is limited by its potential complications such as bleeding and post-procedure pancreatitis, and requires an experienced operator.

Confocal laser endomicroscopy

Needle-based confocal laser endomicroscopy (nCLE) is a novel diagnostic method that allows for real-time optical biopsy at a subcellular resolution during a EUS procedure. The patient is administered with 2.5 mL of 10% fluorescein intravenously, and a confocal mini-probe with a 0.632-mm diameter is preloaded into a 19-gauge EUS needle and locked into position with 2 mm exposed beyond the tip. The images are obtained 6-8 min after fluorescein injection. In solid pancreatic masses, residual regular glandular pancreatic structures on nCLE are characteristic of CP (Table 2). In cystic lesions, the presence of villous epithelial structures on nCLE may be associated with pancreatic cystic neoplasms. A study conducted by Karia *et al*[91] compared the interobserver agreement and diagnostic accuracy of nCLE *via* FNA for pancreatic cystic lesions (PCLs) and found that the diagnostic accuracy for PCLs was low, with a mean sensitivity of 46% and an unimpressive agreement (k = 0.13). Another study found that nCLE is a safe and feasible diagnostic method for cystic neoplasms with an overall accuracy of 80% (sensitivity: 66%, specificity: 100%)[92]. Another study reported a sensitivity of 90.3% and specificity of 89.5% for the diagnosis of PDAC *via* nCLE and a sensitivity of 94.3% and specificity of 98.1% for the diagnosis of PCLs *via*

nCLE ($P < 0.05$; Table 2)[93]. These results suggest that nCLE is a promising method that can improve the diagnostic efficiency of EUS technology by providing better imaging accuracy and verifying EUS imaging features by combining subcellular observation with tissue acquisition. Furthermore, this method can be a complementary modality for detecting the subtle changes of early CP and distinguishing malignancies from benign lesions. Limitations of nCLE include the potential complications of infections, bleeding, and pancreatitis. Furthermore, patients with significant coagulopathy or allergy to fluorescein should not undergo nCLE and FNA.

CONCLUSION

No individual diagnostic method can be used to establish a diagnosis of early CP due to the non-specific clinical presentation and subtle morphological changes as well as the lack of globally accepted standards. Various advanced modalities have been used to improve the accuracy of the diagnostic methods for CP. It is important to consider the possible aetiologies, risk factors, and complications of the disease in patients suspected to have CP. Inflammatory complications are mainly due to alcohol consumption and fibrotic complications are mainly due to smoking. Pancreatic insufficiencies have been associated with disease duration and age at onset[94]. CP is typically diagnosed using imaging modalities that can detect morphological changes in the pancreas or detect functional insufficiency (by combining imaging and secretin stimuli). Elastography can quantitatively evaluate the stiffness of the pancreatic parenchyma. However, each imaging modality has limitations that require verification of the diagnosis using an additional modality. Furthermore, there is a lack of consistency among the features and diagnostic criteria of different imaging modalities. The diagnostic criteria of MRCP and ERCP are based on the Cambridge scoring system, which focuses on the morphology of the MPD and the appearance of its side branches; however, a lack of strong concordance between the ERCP- and MRCP-based grading systems for CP using the Cambridge criteria has been recently reported[8,95,96]. Therefore, the imaging standards for CP may need to be revised to improve the consistency of the diagnostic methods. Prospective studies are required to verify the diagnostic accuracy of these imaging modalities. However, ERCP purely for diagnostic purposes for assessing chronic pancreatitis is outdated with risks greater than benefits and should not be done. Each imaging modality has sensitive indicators for pancreatic pathological changes, and some modalities do not have a high sensitivity or specificity for pancreatitis at an early stage, as shown in Table 2. Promising tools, such as nCLE, are currently being developed and require more studies for the verification of their diagnostic efficiency and potential. Furthermore, probable biomarkers for the diagnosis of early CP or to rule out malignancy, such as metabolomic and microRNA signatures in the blood, have been investigated, but they also require further verification[97-100].

A limitation of this review is that it could not identify a single appropriate modality for the diagnosis of early CP; however, comparisons of these clinical approaches provide references for physicians to help establish a diagnosis of early CP. The modalities used may depend on the local availability of a particular test, *e.g.*, pancreatic function testing. MRCP or EUS may not be universally available at each institution. In conclusion, although several imaging modalities can elucidate specific pancreatic features, an accurate and definite diagnosis of early CP should be based on the patient's risk factors, symptoms, imaging results, and histological findings when available. There has been a renewed recent interest in the use of artificial intelligence (AI)[101] in medical imaging and application of AI to EUS images and the other modalities discussed above could potentially further increase the reliability in diagnosis of CP since medical diagnosis based on images is subject to interpretation of the reader and interobserver variability can become a greater issue even more for the diagnosis of an early stage of disease than a late one.

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MINIREVIEWS

Dysregulated liver function in SARS-CoV-2 infection: Current understanding and perspectives

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Abstract

Since it was first reported in December 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has spread rapidly around the world to cause the ongoing pandemic. Although the clinical manifestations of SARS-CoV-2 infection are predominantly in the respiratory system, liver enzyme abnormalities exist in around half of the cases, which indicate liver injury, and raise clinical concern. At present, there is no consensus whether the liver injury is directly caused by viral replication in the liver tissue or indirectly by the systemic inflammatory response. This review aims to summarize the clinical manifestations and to explore the underlying mechanisms of liver dysfunction in patients with SARS-CoV-2 infection.

Key Words: SARS-CoV-2; COVID-19; Dysregulated liver function; Cytokine storm

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Core Tip: The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection is one of the most influential emerging infectious diseases worldwide. Accumulating evidence suggests

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that liver injury is common in COVID-19 patients, and many severe cases tend to be associated with dysregulated liver functions. In this review, we summarize the currently available data of liver enzyme abnormalities in patients confirmed to have COVID-19 and analyze multiple risk factors for liver injury. However, the mechanism of liver impairment seems to be multifactorial. The evidence of direct liver injury triggered by SARS-CoV-2 infection or indirect liver injury induced by overwhelmed cytokine storm will also be discussed.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) has broken out worldwide and was declared as a global pandemic by the World Health Organization (WHO) on March 11, 2020 after it was first reported in December 2019. The virus was later isolated[1] and was named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by the International Committee for Taxonomy of Viruses. To date, this virus has spread to 220 countries, infecting 179601602 people with 3891974 deaths (as of June 24, 2021, source: Johns Hopkins University; https://coronavirus.jhu.edu/map.html), which constitutes a public health emergency of international concern.

The severity of symptoms in patients with COVID-19 ranges from mild self-limited respiratory disease to severe progressive pneumonia and respiratory failure, with an overall mortality rate of 2.3%[2]. The typical clinical manifestations of COVID-19 include elevated body temperature (> 37.3 °C), dry cough, dyspnea, leukocytosis, lung infiltration, and no significant improvement after antibiotic treatment for 3 d[3]. In addition to the respiratory system, various organs are reported to be involved in the process of SARS-CoV-2 infection, which caused complications that aggravate the infection[4]. Up to 50% of COVID-19 patients have been reported to have abnormal liver biochemical indicators, including elevated expression of aminotransferases, glutamyl transferase, and alkaline phosphatase (ALP)[3,5,6], and the proportion of infected patients with abnormal liver functions is greater in highly epidemic areas than other regions[7]. In addition, 58%-78% of patients with severe COVID-19 have apparent liver damage. Liver injury in turn increases the risk of other outcomes, such as cardiac arrest, requirement for intubation, acute respiratory distress syndrome, arrhythmia and shock[8]. Therefore, it is crucial to understand the mechanisms of the aberrant liver functions caused by this novel respiratory virus[9]. It is currently believed that three mechanisms may account for the liver damage in COVID-19 patients: (1) Hepatic bile duct endothelial cells and hepatocytes are directly infected by SARS-CoV-2 mediated by the cellular receptors; (2) A cytokine storm is induced by overactivated immune responses and causes inflammatory liver damage; and (3) Multiple risk factors such as underlying liver diseases may cause secondary liver damage following SARS-CoV-2 infection. In this review, we summarize the clinical manifestations and explore the potential underlying mechanisms of liver dysfunctions in patients with SARS-CoV-2 infection.

SARS-CoV-2: GENOME STRUCTURE AND TRANSMISSION

Coronaviruses (CoVs) are a group of enveloped positive single-stranded RNA viruses that belong to the Coronaviridae family of order Nidovirales[10]. CoVs are further grouped into α-, β-, γ-, and δ-coronaviruses. Among them, α- and β-CoVs can infect mammals and γ-coronaviruses infect birds and δ-coronaviruses infect both[11]. SARS-CoV-2, initially isolated from human airway epithelial cells, was identified as the seventh member of the CoVs capable of infecting humans. It genetically belongs to the β-CoV genus in the same clade with SARS-CoV and middle-east respiratory syndrome

coronavirus (MERS-CoV)[1,12].

Genome structure and variability

The SARS-CoV-2 genome shares around 76.5% amino acid sequence identity with SARS-CoV[13] , which might be responsible for the shared transmission mode and pathogenesis of these two viruses. The SARS-CoV-2 genome contains 14 open reading frames, encoding 27 proteins (Figure 1A). Four major structural proteins are encoded by the 3'-terminus of the genome, including the spike surface glycoprotein (S), matrix protein (M), small envelope protein (E), and nucleocapsid protein (N)[11] (Figure 1B). The S protein initiates the infection by interacting with the cellular receptor through its receptor binding domain (RBD). SARS-CoV-2 infects the target cells by S protein on the surface of the virion binding to cellular angiotensin-converting enzyme 2 (ACE2) receptors followed by fusion of the cellular and viral membranes (Figure 1C). In this process, transmembrane protease serine 2 (TMPRSS2), together with the endosomal cysteine proteases cathepsin B and L (CatB/L), are used for S protein priming. The S protein of SARS-CoV-2 is efficiently cleaved at the junction of S1 and S2 proteins to produce an S2 subunit to facilitate SARS-CoV-2 entry[14]. Considering the major role played in the pathogenesis, S protein is considered to be a potential target for vaccine design and drug development[9]. E protein is involved in the assembly and release of virions. As the most abundant viral protein, M protein plays an important role in RNA packaging[15] and N protein mediates the transcription and replication of viral RNA [16].

The variability of the SARS-CoV-2 genome is lower when compared to other human CoVs. There were 149 mutations in 103 sequenced SARS-CoV-2 genomes in the early stage of the pandemic[17]. The virus was classified into two major variants (L and S) through population genetic analysis. The L type, derived from the S type of SARS-CoV-2, was reported to be evolutionarily more aggressive[17]. Recently, a more transmissible phylogenetic cluster (named lineage B.1.1.7), which has 23 specific genetic changes in S protein of SARS-CoV-2, was reported in the UK, and rapidly became the dominant strain in London. Mutation N501Y affects the structure of the RBD and P681H is adjacent to the furin-cleavage site, which increases the binding affinity of the virus to ACE2 receptors by 1000-fold. Moreover, the deletion 69-70del on the S protein facilitates viral evasion from immune surveillance. Collectively, these variations have raised concerns about potential antigenic drift of SARS-CoV-2 similar to that of influenza virus[18].

Transmission of SARS-CoV-2

SARS-CoV-2 is a zoonotic virus that spreads in humans through respiratory droplets [19]. Comparative sequence analysis of the SARS-CoV-2 genome has revealed a striking similarity with bat CoVs, indicating that bats may be the origin of this virus [20,21]. Although many COVID-19 cases initially reported in China were traced to have connection with Huanan Seafood Wholesale Market in Wuhan[6,22,23], several reports clearly indicate that the global spread of the SARS-CoV-2 might have preceded the discovery of the first case in Wuhan. The epidemic likely started between October 6 and December 11, 2019 in the USA, Italy and France[24,25];however, debate still exists about the source of SARS-CoV-2. Since the transmission of SARS-CoV-2 needs intermediate hosts, pangolins have been suggested as a probable intermediate host [26]. In February 2020, evidence of human-to-human transmission of SARS-CoV-2 was first reported[27]. Thereafter, a case of SARS-CoV-2 provided proof that human-to-human transmission occurred during the incubation period[28]. With the appearance of the second-generation patients and many medical staff in the hospital being infected, the strong infectious ability of SARS-CoV-2 was confirmed[29]. With the detection of SARS-CoV-2 RNA in fecal sample from a COVID-19 patient in the USA, SARS-CoV-2 could also be transmitted through the fecal–oral route[30]. Since September 2020, the route of transmission from contaminated objects to people has been reported by tracing the origin of local confirmed cases in Qingdao, Tianjin, Dalian, Chengdu and other cities in China, further complicating epidemic prevention and control.

DYSREGULATED LIVER FUNCTION IN COVID-19 PATIENTS

The common clinical manifestations of patients with COVID-19 are cough, fever, shortness of breath, and other respiratory symptoms[31]. Gastrointestinal symptoms including nausea, vomiting, and diarrhea are also observed in some patients. The

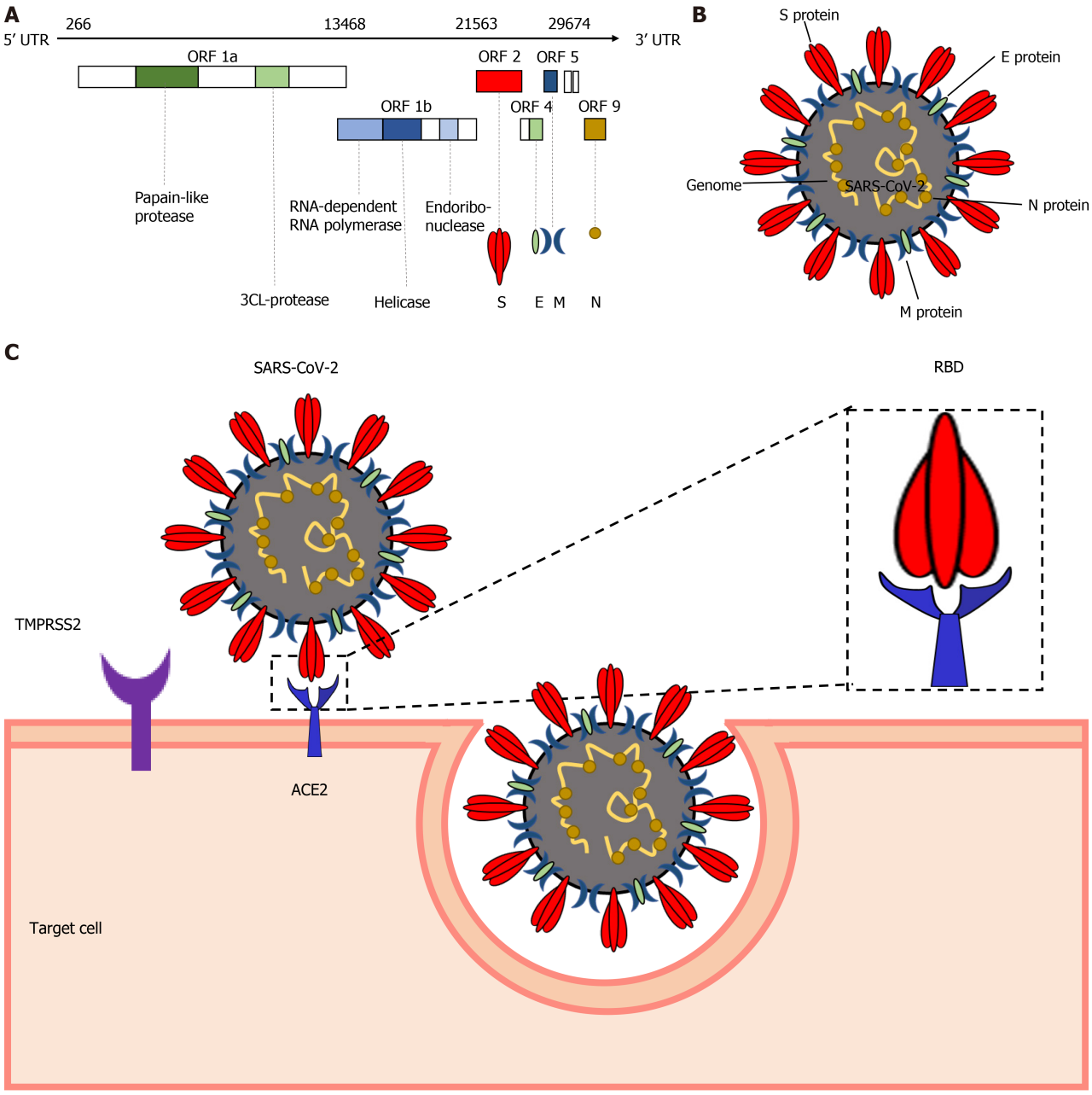


Figure 1 Severe acute respiratory syndrome coronavirus 2 genome and its pathogenesis. A: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) genome structure and its encoded proteins; B: Virion structure of SARS-CoV-2 illustrating viral structural proteins including spike protein (S), envelope protein (E), membrane protein (M), and nucleocapsid protein (N); C: SARS-CoV-2 entry to the target cells by binding the receptor-binding domain of S protein to cellular receptors, such as angiotensin-converting enzyme 2 and transmembrane protease serine 2. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; RBD: Receptor-binding domain; TMPRSS2: Transmembrane protease serine 2; ACE2: Angiotensin-converting enzyme 2.

abnormal values of liver enzymes have frequently been noted in patients with COVID-19. Existing data suggest that, in most cases, liver enzyme elevations are mild to moderate and rarely severe. Limited pathophysiological studies have indicated that SARS-CoV-2 infection can cause liver injury, although SARS-CoV-2-induced liver injury is not the leading cause of death.

Altered biochemical markers

Liver injury in hospitalized patients is mainly manifested by changes in biochemical liver markers. Many studies have reported the presence of liver dysfunction in COVID-19 patients (Table 1). Respiratory failure is a common characteristic in severe cases, characterized by an imbalance of oxygen supply leading to an increase in transaminases[32]. As reported, 14%-53% of patients have elevated aspartate aminotransferase (AST), alanine aminotransferase (ALT), g-glutamyltransferase (GGT), hypoalbuminemia, and slightly elevated bilirubin[33]. The elevation of AST (39.4%) is more

Table 1 Altered biochemical markers of patients with coronavirus disease 2019

Region	No. of patients	Rate of abnormal liver test	Hepatocyte injury markers		Bile duct injury or cholestasis markers		Hepatic clearance markers	Infection-related biomarkers	Refs.
			ALT	AST	ALP	GGT	Total bilirubin	CRP	
Shenzhen, China	417	76.3%	23.4%	14.80%	N/A	24.4%	11.5%	N/A	[33]
Wuhan, China	115	N/A	9.57%	14.78%	5.21%	13.4%	6.96%	N/A	[34]
Wuhan, China	99	43%	28%	35%	N/A	N/A	18%	86%	[6]
Wuhan, China	1099	N/A	21.3%	22.20%	N/A	N/A	10.5%	60.7%	[31]
Shanghai, China	148	37.20%	18.2%	21.6%	4.10%	17.60%	6%	8.7-32.3%	[35]
Wuhan, China	69	N/A	33%	28%	N/A	N/A	N/A	67%	[41]
Fuyang, China	125	N/A	20.8%	21.60%	N/A	N/A	N/A	70.4%	[42]
Japan	22	68.20%	54.5%	N/A	N/A	54.50%	N/A	N/A	[36]
Turkey	554	N/A	27.6%		4%		N/A	N/A	[37]
Zaragoza, Spain	531	64.3%	28.6%	40.90%	N/A	47.30%	N/A	N/A	[38]
Wuhan, China	81	N/A	29.5%	17.90%	N/A	N/A	3.6%	41.8%	[43]
New York, United States	5700	N/A	39%	58.40%	N/A	N/A	N/A	6.4-26.9%	[44]

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: -glutamyltransferase; CRP: C-reactive protein; N/A: Not available.

significant than that of ALT (28.1%) in severe cases. Among these markers affected by viral infections, elevated AST is related to a higher risk of death, while elevated GGT [33-38], ALP[34-36], together with bilirubin levels[6,31,33-35], are associated with biliary tract damage. Some patients have hypoalbuminemia, an independent predictor of mortality, which may be related to inflammation and poor prognosis[39,40]. Some studies have also reported C-reactive protein (CRP) elevation in many severe COVID-19 cases[6,31,35,41-44], indicating that overactivation of the immune system might account for disease severity, which can be used to predict worse outcomes in COVID-19 patients. In the elderly population with underlying liver disease, liver enzymes, especially transaminases, are elevated due to lobular necrosis[45].

Pathophysiological characteristics of liver tissues in COVID-19 patients

Mild portal vein inflammation, moderate microvesicular steatosis, and necrotizing inflammation have been observed in liver autopsy tissue of COVID-19[10]. This is likely due to increase of monocyte chemoattractant protein 1 (MCP-1) in patients infected with SARS-CoV-2, which aggravates fatty hepatitis[46]. Autopsy results from COVID-19 patients in Wuhan have shown infiltration of monocytes and lymphocytes in the portal vein area, accompanied by hepatic sinus congestion and microthrombus formation[45]. Cytologically, hepatocyte degeneration with focal lobular necrosis and neutrophil infiltration are observed together with swollen mitochondria, endoplasm reticulum expansion, and decreased glycogen particles in the hepatocytes. The histological manifestations are apoptosis and binuclear hepatocytes. In addition, immunohistochemical results have shown a lack of $CD4^+$ cells and $CD8^+$ T cells in liver tissue, suggesting that immunopathological insult may not be the main factor leading to liver injury.

POTENTIAL MECHANISMS OF LIVER INJURY IN COVID-19 PATIENTS

SARS-CoV-2 initiates membrane fusion and cytoplasmic invasion by entering respiratory endothelial cells expressing ACE2 and TMPRSS2, triggering an initial immune response characterized by inflammatory cytokine production. Afterwards,

the downstream proinflammatory immune responses of pathogenic Th1 cells and monocyte signaling pathways are activated[47]. Two hypotheses have been proposed to explain the mechanisms of liver dysfunctions in COVID-19 patients: direct injury by SARS-CoV-2[48] and indirect injury from a cytokine storm.

Direct effects of SARS-CoV-2 infection on liver injury

It has been reported that SARS-CoV-2 infection of various types of liver cells causes liver injury through direct cytopathic effects[48]. As a functional receptor for SARS-CoV-2 S protein binding, ACE2 is widely expressed on the surface of various human cells. Due to the low expression level of ACE2 in liver tissue (approximately 0.31%), SARS-CoV-2 was once considered unlikely to infect the liver. However, Li *et al*[49] systematically evaluated the expression of host receptor genes interacting with SARS-CoV-2 in liver tissue and their distribution patterns in different cell types using single-cell transcriptomics. ACE2 protein is expressed in various cell types in the liver, especially in bile duct cells[50], likely leading to direct injury to the liver (Figure 2). Boettler *et al*[51] proved that SARS-CoV-2 has a stronger affinity for bile duct cells than hepatocytes because of higher ACE2 receptor expression.

It has been proposed that SARS-CoV-2 infection of bile duct cells is the source of viral nucleic acid detection in feces[51]. Even in patients with negative throat swabs for viral RNA, 48% of feces were positive for SARS-CoV-2 RNA, which may be derived from portal vein viremia. Although SARS-CoV-2 can infect bile duct cells, the histological characteristics of bile duct injury have not been observed. Wang *et al*[48] observed many SARS-CoV-2 virions in the hepatocyte cytoplasm of two cases with COVID-19. Most of the virosomes were found to have complete envelopes with crown protrusions, providing evidence that SARS-CoV-2 can enter and replicate in hepatocytes. However, the low expression level of ACE2 on hepatocytes cannot fully explain the hepatotropic nature of SARS-CoV-2. One possibility is that there are alternative receptors or co-receptors other than ACE2. This hypothesis has gradually been confirmed by the discovery of more co-receptors that facilitate SARS-CoV-2 entry. For example, tyrosine-protein kinase receptor UFO (AXL), heparan sulfate, scavenger receptor B type 1 (SR-B1), neuropilin-1 (NRP1) and CD147 have been identified as attachment factors for SARS-CoV-2 infection[52-57], although the abundance of these co-receptors in liver tissue remains to be determined. The other possibility is that the expression of ACE2 in hepatocytes is temporarily upregulated when the virus enters the cells, assisting infection. Again, more evidence is needed to support this hypothesis.

Indirect injury caused by cytokine storm

The variable symptoms in severe and asymptomatic patients with COVID-19 suggest that host immune responses contribute to the pathogenesis. SARS-CoV-2 infection leads to excessive production of a series of cytokines by macrophages and neutrophils known as a cytokine storm[47], which is one of the main features in COVID-19 patients and may be a key factor affecting disease severity and mortality. Cytokines are self-antigens that induce autoimmunity, and could account for the multisystem inflammatory syndrome often seen in COVID-19 patients. $CD4^+$ and $CD8^+$ T cells in the peripheral blood of COVID-19 patients are highly activated *via* Toll-like receptors. Elevated proinflammatory $CD4^+$ T cells and cytotoxic granular $CD8^+$ T cells suggests an antiviral immune response and excessive activation of T cells in the peripheral blood[10]. The host cells are attacked by activated T cells leading to necrosis and apoptosis, and many damage-related pattern molecules are involved to amplify the inflammatory signals. Lymphopenia is also observed in the disordered cytokine storm, but it is still unknown whether it is caused by destruction of lymphocytes or tissue infiltration. It has been demonstrated that the proinflammatory cytokines are elevated significantly in patients with severe COVID-19, leading to severe pneumonia. The inflammatory cytokines not only protect the host cells from injury but also cause blood clotting, thereby blocking the vessels and resulting in decrease of oxygen saturation and sepsis, which is considered to be one of the causes of insufficient lung ventilation. Therefore, hypoxic hepatitis is common in patients with severe COVID-19, especially elderly patients with right congestive heart failure who are prone to have hypoxic–ischemic liver injury[48].

More specifically, the cytokine storm induced by SARS-CoV-2 is characterized by high expression of interleukin (IL)-6 and tumor necrosis factor-α (TNF-α). Activated pathogenic Th1 cells produce proinflammatory cytokines, such as granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-6. GM-CSF in turn stimulates $CD4^+$ T cells to secrete large quantities of cytokines, such as TNF-α, IL-10, IL-6 and type I interferons[58] (Figure 2). The inflammatory response is obvious in COVID-19

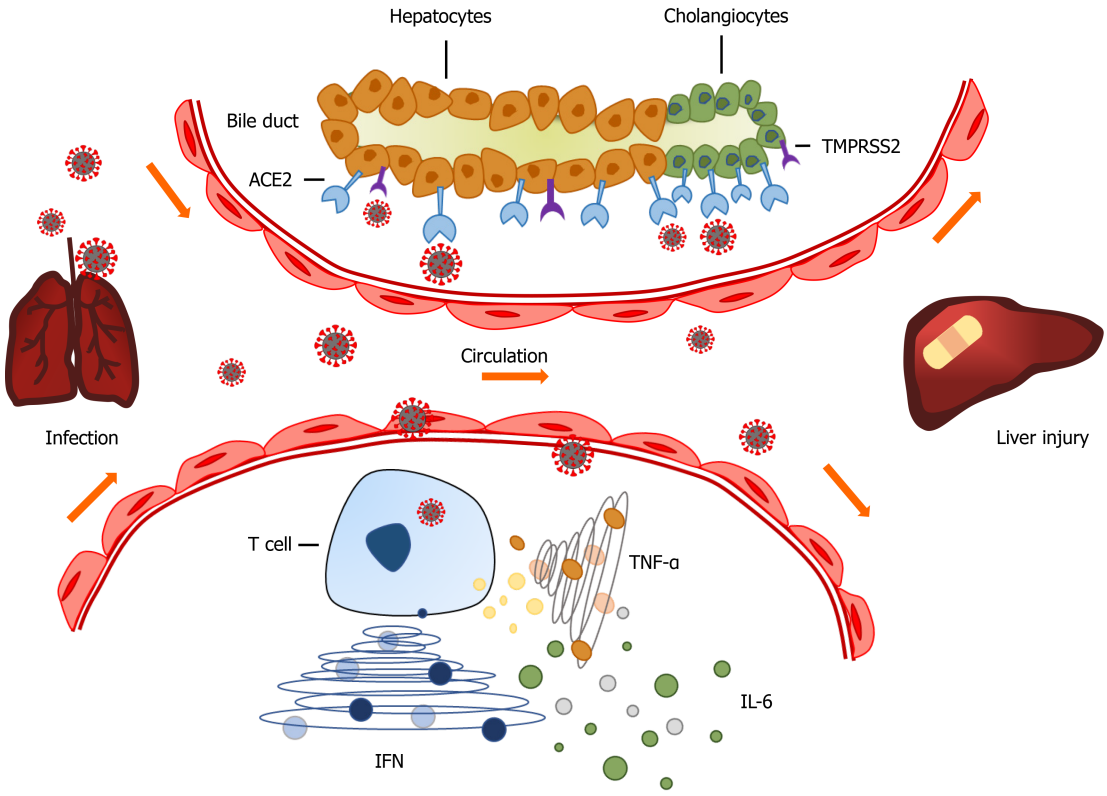


Figure 2 Schematic diagram of the two mechanisms of severe acute respiratory syndrome coronavirus 2-induced liver dysfunction. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes direct liver injury through binding to the angiotensin-converting enzyme 2 receptor and transmembrane protease serine 2 expressed on cholangiocytes and hepatocytes. Indirect injury by cytokine storm. T cells are stimulated to secrete large quantities of cytokines including type I interferons, interleukin-6, and tumor necrosis factor-α following SARS-CoV-2 infection, leading to systemic excessive inflammation syndrome. ACE2: Angiotensin-converting enzyme 2; TMPRSS2: Transmembrane protease serine 2; TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6; IFN: Interferons.

patients as indicated by increased expression of inflammatory biomarkers such as lactate dehydrogenase (LDH), IL-6, IL-2, CRP, serum ferritin, and D-dimer[59-61]. It has been reported that 28% of patients who died of multiple organ damage, including liver failure, was due to an excessive inflammatory response. In the case of liver injury, ACE2 expression is upregulated due to the compensated proliferation of hepatic parenchymal cells derived from bile duct epithelial cells, which increases the opportunities of viral infection in liver tissue.

Neutralizing cytokine release syndrome (CRS) that blocks the signal transduction pathway of these cytokines contributes to the development of effective drugs for the treatment of patients with severe COVID-19, and targeting CRS has significant implications for reducing mortality. One such example is tocilizumab, an IL-6 inhibitor, which improves the condition of patients within 15 d and reduces mortality considerably[62]. Although the cytokine storm is involved in liver injury, these cytokines also function in viral suppression. Therefore, application of treatment regimens to block cytokine signal transduction must be careful. Clinically, the application of synthetic corticosteroid dexamethasone worsens outcomes in milder COVID-19 patients, but reduces mortality in severe cases[63]. Likewise, other studies have supported that immunosuppression has a beneficial effect if given at a late stage, whereas immunostimulation enhances antiviral activity in the early stage[64], indicating that the timing of treatment and the specific patient population need to be identified to personalize the therapeutic schemes.

Liver injury caused by other factors in COVID-19 patients

Current evidence is insufficient to conclude that liver injury in COVID-19 patients results entirely from SARS-CoV-2 infection. Considering the multisystem involvement in severe COVID-19 patients, liver injury may be caused by multiple factors, including drug toxicity, systemic inflammation, liver congestion, microvascular thrombosis, or damage induced by cytotoxic T cells and innate immune responses following SARS-CoV-2 infection.

Drug toxicity: Currently there is no specific treatment for COVID-19, although initial clinical guidelines recommended using antiviral drugs and monoclonal antibodies such as remdesivir, chloroquine, tocilizumab, lopinavir, ritonavir, and traditional Chinese medicine. However, it has been reported that both lopinavir and ritonavir have little clinical effect on COVID-19 patients[65]. According to WHO and National Health Commission of China guidelines, patients with moderate symptoms should receive timely treatment with *Coriolus versicolor*, abidor, chloroquine phosphate, and recombinant human interferon α-2b. For severe/critical patients, respiratory support, appropriate hormone therapy, and traditional Chinese medicine may be beneficial [66]. Chloroquine phosphate has been proved to cause significant liver damage[67]. A more recent study reported that ACE inhibitors and angiotensin II receptor blockers might contribute to liver impairment in COVID-19 patients, although more studies are needed to confirm these findings[33]. In addition, fever is one of the main symptoms for COVID-19 patients. Paracetamol, which is a commonly used antipyretic drug, has been proven to cause fulminant liver failure, so the possibility of excessive use of paracetamol leading to elevated ALT cannot be ruled out[59,68].

Liver congestion: Forty percent of patients are at high risk of venous thromboembolism (VTE) when admitted to hospital. High-risk VTE patients have elevated expression of ALT, AST and CRP[69]. It is well known that viral infection leads to a hypercoagulable state, increasing the risk of thromboembolism[70,71]. The incidence of VTE is 20% on day 7 and 42% on day 21, even among hospitalized patients taking anticoagulants[72]. Recent studies have found that in some younger patients, microvascular thrombosis causes end-stage organ damage and contributes to liver injury. Anticoagulant drugs can be used to prevent the development of VTE. A few studies have concluded that 80% of COVID-19 patients with cirrhosis and portal hypertension received anticoagulant therapy, such as low-molecular-weight heparin, and those patients do not have serious bleeding complications[73,74]. However, Wang *et al*[69] have reported that 11% of COVID-19 patients with high risk of VTE also have a high risk of bleeding. Therefore, evaluating the risk of thromboembolism and rational use of anticoagulants for the treatment of COVID-19 patients is critical.

Underlying liver diseases: Chronic liver diseases, including chronic viral hepatitis and nonalcoholic fatty liver disease (NAFLD), affect approximately 300 million people in China and constitute a major global burden of disease. Although chronic liver disease does not increase the infection risk of patients with SARS-CoV-2 in general, patients with liver cirrhosis or hepatocellular carcinoma tend to be more susceptible to SARS-CoV-2 infection due to systemic immunodeficiency. Furthermore, the underlying liver diseases may worsen disease progression in patients with COVID-19. A study consisting of > 17 million people in the United States found that although < 1% of the confirmed COVID-19 cases had chronic liver diseases, chronic liver disease was an independent risk factor for death from SARS-CoV-2 infection[75].

Hepatitis B virus (HBV) does not predispose COVID-19 patients to more severe outcomes, although HBV patients infected with SARS-CoV-2 normally present with aggravated liver damage together with more severe thrombocytopenia and monocytopenia, coagulation dysfunction, and more disturbed hepatic function in relation to albumin production and lipid metabolism[76], and these co-infected patients have a tendency to HBV reactivation[67]. Astrilizumab and baritinib, which are used clinically to antagonize adverse immune reactions, tend to induce HBV reactivation, leading to impaired liver function in HBV patients[77]. However, it is currently unclear whether viral infection aggravates cholestasis in patients with the cholestatic disease. Further in-depth research should focus on persistent liver damage and active viral replication in HBV patients after co-infection with SARS-CoV-2. Recent studies have demonstrated the possible impact of SARS-CoV-2 infection on NAFLD. It has been shown that SARS-CoV-2 infection increases the possibility of NAFLD progressing to nonalcoholic hepatitis. ACE inhibitors are commonly used in the anti-inflammatory and anti-obesity treatment of NAFLD. Although there have been no reports on the correlation between the use of ACE inhibitors and mortality, one study has shown that ACE inhibitors upregulate expression of ACE2 receptor, thereby increasing the viral entry in patients taking such drugs[78].

How the underlying liver diseases affect liver function in patients with COVID-19 remains elusive. Whatever mechanisms are involved in liver damage, we need to be more cautious in managing COVID-19 patients with underlying liver diseases. For example, the British Liver Foundation recommends that patients taking steroids or immunosuppressive drugs maintain strict social distance[79]. The European Association for Liver Research and the European Association for Clinical Microbiology

and Infectious Diseases recommend priority outpatient visits, prehospital assessment of risk factors, reduction of exposure through modification of waiting areas, reduction of waiting times, and use of online system to order medication.

CONCLUSION

Although liver dysfunction is common in patients with COVID-19, the detailed mechanisms remain incompletely understood. Available evidence suggests that the severity of liver damage is partly due to direct infection by SARS-CoV-2 to the liver and partly due to the cytokine storm produced by an overactive immune response. In addition, multiple risk factors, such as drug toxicity and liver congestion, need to be taken into account. For COVID-19 patients with underlying liver diseases, the causes of liver damage are more complex and more personalized management should be considered. Future directions should focus on deciphering the virus–host interactions to better understand the detailed molecular mechanisms of the liver impairment following SARS-CoV-2 infection. In the meantime, rapid development of effective preventive vaccines and specific antiviral drugs is critical to control this ongoing pandemic.

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MINIREVIEWS

Impact of surgery for chronic pancreatitis on the risk of pancreatic cancer: Untying the Gordian knot

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Abstract

Pancreatic ductal adenocarcinoma is an aggressive tumor with poor long-term outcomes. Chronic pancreatitis (CP) is considered a risk factor for the development of pancreatic cancer (PC). Persistent pancreatic inflammation and activation of pancreatic stellate cells play a crucial role in the pathogenesis of CP-related PC by activating the oncogene pathway. While genetic mutations increase the possibility of recurrent and persistent pancreatic inflammation, they are not directly associated with the development of PC. Recent studies suggest that early surgical intervention for CP might have a protective role in the development of CP-related PC. Hence, the physician faces the clinical question of whether early surgical intervention should be recommended in patients with CP to prevent the development of PC. However, the varying relative risk of PC in different subsets of CP underlines the complex gene-environment interactions in the disease pathogenesis. Hence, it is essential to stratify the risk of PC in each individual patient. This review focuses on the complex relationship between CP and PC and the impact of surgical intervention on PC risk. The proposed risk stratification based on the genetic and environmental factors could guide future research and select patients for prophylactic surgery.

Key Words: Chronic pancreatitis; Pancreatic adenocarcinoma; Pancreatic cancer; Surgery; Hereditary pancreatitis; Pancreatitis

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Core Tip: Chronic pancreatitis (CP) is a significant risk factor for pancreatic cancer (PC). However, the relative risk is not as high as previously reported. Persistent pancreatic inflammation plays an important role in pancreatic carcinogenesis, and genetic mutations implicated in the pathogenesis of CP are not directly involved in the development of PC. Although surgery for CP reduces the risk of PC, the evidence is not strong enough to recommend routine prophylactic surgery in CP patients. The risk stratification proposed in this review considers the genetic and environmental factors that could guide future prospective studies and select patients for prophylactic surgery.

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INTRODUCTION

Chronic pancreatitis (CP) is a complex fibro-inflammatory disease of the pancreas characterized by destruction and fibrotic replacement of the pancreatic parenchyma [1]. Pain is the dominant symptom of CP, followed by clinical manifestations of exocrine and endocrine dysfunction[2]. Surgery and endoscopic interventions are primarily indicated in symptomatic CP patients to provide pain relief and may prevent disease progression if done early in the disease course[3,4]. Pancreatic ductal adenocarcinoma, the most common type of pancreatic cancer (PC), is an aggressive tumor with a dismal prognosis[5]. Epidemiological studies have identified CP as a risk factor for PC[6-8]. Recurrent or persistent pancreatic inflammation in the setting of CP has been implicated in pancreatic carcinogenesis[9,10]. Recent studies have shown that surgery for CP reduces the risk of PC[11,12]. This evidence raises the question of whether early surgical intervention should be considered in patients with CP to prevent the development of PC. The proposed mechanistic definition for CP highlights the complex gene-gene and gene-environmental interactions in the disease pathophysiology[13]. As not all patients with CP develop PC, and a high proportion of patients with PC do not have a history of CP, this review focuses on the complex relationship between CP and PC and the impact of surgical intervention on the risk of PC.

SEARCH STRATEGY

A PubMed search of relevant articles was performed by three authors The reference lists of the articles also were searched for additional appropriate studies. The keywords and combinations included in the search were: "pancreatitis"; "chronic pancreatitis"; "hereditary pancreatitis"; "pancreatic cancer" and "chronic pancreatitis"; "pancreatic cancer" and "hereditary pancreatitis"; "pancreatitis" and "pancreatic cancer"; "genetics" and "pancreatic cancer"; "pancreatic ductal adenocarcinoma" and "pancreatitis"; "genetics" and "chronic pancreatitis" and "pancreatic cancer"; "surgery" and "chronic pancreatitis" and "pancreatic cancer". The search was limited to publications in English and stressed the latest evidence. All the authors agreed that the articles selected for review were relevant.

CP AND PC

Incidence of PC in CP

It is essential to understand the incidence of PC in CP patients to determine the impact of surgery for CP on the risk of PC. In the 19th century, Rudolph Virchow observed white blood cells within tumor tissue and suggested a relationship between inflammatory pathology and cancer[14]. Well known associations that support the hypothesis of inflammation-induced metaplasia and carcinogenesis include inflam-

matory bowel disease and colorectal cancer, gallstones and gallbladder cancer, Barrett's esophagus, and esophageal cancer[15]. The earliest report of an association between CP and PC dates to the 1950s, when an autopsy study identified CP changes in 49% of 100 patients who died of PC[16]. Since then, multiple studies have documented an increased risk of PC in CP patients[17-19]. Raimondi *et al*[6], in a meta-analysis of 22 case-control and cohort studies, reported a relative risk (95%CI) of 13.3 (6.1-28.9), which means that CP patients had 13.3 times more risk of developing PC compared with the general population. However, the included studies did not consider the lag period, *i.e.* the interval between CP and PC diagnosis. To be labeled as CP-related PC, it is recommended to exclude PC cases diagnosed within 2 years of CP diagnosis to avoid potential misclassification. After considering the lag period, the relative risk of CP-related PC dropped to 11.8 with a 1-year lag period and to 5.8 with a 2-year lag period (Table 1). Tong *et al*[7] in a meta-analysis of 17 studies with acute, chronic, and unspecified pancreatitis, reported that CP patients had a 10.3-fold risk of developing PC compared with the general population. When the association between CP and PC was stratified by time, they reported risk estimates (95%CI) of 23.3 (14.0-38.9), 3.03 (2.41-3.81), 2.82 (2.12-3.76), and 2.25 (1.59-3.19) for lag periods of less than 1, 2, 5, and 10 years, respectively. While the estimated risk was less than reported in the previous meta-analysis, the inclusion of acute and unspecified pancreatitis along with CP might have underestimated the association between CP and PC, as acute pancreatitis is less likely to progress to PC compared with CP. Kirkegård *et al*[8], in a recent meta-analysis of 13 CP studies, estimated 8-fold and 3.5-fold increased risks of PC at 5- and 9-year lag periods, respectively, from CP diagnosis to PC diagnosis (Table 1). The meta-analyses suggest that patients with CP are at increased risk of developing PC compared with the general population. However, the estimated risk was much lower than that reported in some individual studies, especially when a 2-year lag period was included. While the increased duration of CP-related inflammation is expected to increase PC risk, the decrease in PC risk between the 2- and 5-year lag periods in the meta-analyses suggests initial misclassification of PC as CP in some of the included studies. Also, most studies did not evaluate the impact of confounding risk factors like smoking or alcohol intake on the risk estimate of CP-related PC.

Risk factors for the development of PC in CP patients

The current evidence suggests that the incidence of PC in CP patients is variable (2.7- to 13.3-fold increased risk) and influenced by multiple factors[20,21]. Hence, identifying demographic and clinical risk factors for PC in CP patients will help to determine the subgroup of CP patients who might benefit from surgical intervention. A retrospective analysis of 1415 patients from an Indian center reported that a smoking and nonalcoholic CP etiology was a risk factor for developing PC[22] (Table 2). Among the nonalcoholic CP etiologies, idiopathic senile chronic pancreatitis (ISCP), defined as an age of onset of CP of more than 35 years, had a greater risk of PC than idiopathic juvenile CP (5% *vs* 0.2% at 10 years of follow-up). An inverse association between exocrine insufficiency and malignancy risk indicated that steatorrhea and malignant transformation occurred independently. A retrospective analysis of 2037 CP cases in a patient database in China did not find a significant difference in PC risk between alcoholic and idiopathic pancreatitis[23]. However, the analysis did not include a separate risk analysis of patients with IJSP and ISCP. A single-center European study by Müllhaupt *et al*[24] prospectively evaluated the risk of PC in 332 CP patients. The risk of PC was greater in idiopathic juvenile chronic pancreatitis (IJCP) compared with other CP etiological subtypes. The authors concluded that early onset and prolonged disease was a significant risk factor for CP-related PC. In another European study from Sweden, Vujasinovic *et al*[25] evaluated the risk of PC in a retrospective analysis of prospectively collected pancreatic clinical data. In contrast to other studies, they subclassified the CP patients into eight subgroups and calculated the PC risk. Among CP patients, those with diabetes mellitus and a high body mass index (BMI) at diagnosis or patients with a low BMI and pancreatic exocrine insufficiency at diagnosis were identified as groups at risk of developing CP-related cancer. However, relatively few patients (6/581) developed PC 2 years after the CP diagnosis. Studies conducted in different regions of the globe highlight differences in the demographic and clinical risk factors for PC in different CP populations. To evaluate the reason for the diverse predictive factors, the pathogenesis of CP and pathways for PC in those patients have to be well understood.

Inflammatory and oncogene pathways of PC in CP patients

An accepted explanation of the pathogenesis of CP involves a sentinel acute pancreatitis event that initiates an inflammatory cascade in a susceptible individual[26,27]

Table 1 Systematic reviews and meta-analyses evaluating the association between chronic pancreatitis and pancreatic cancer

Ref.	Number of studies	Number of patients	Risk estimate	Remarks
Raimondi *et al*[6]	22 (12 case-control, 10 cohort)	16124	RR, 11.8 (95%CI: for 1-yr lag period; RR, 5.8 (95%CI: 2.1-15.9) for 2-yr lag period; RR, 69.0 (95%CI: 56.4-84.4) for hereditary pancreatitis; RR, 100 (95%CI: 37.0-218.0) for tropical pancreatitis	Details of seven studies included for risk estimate calculation not mentioned; Details of number of patients with pancreatic cancer in included studies not mentioned; High relative risk reported for tropical pancreatitis based on a single study
Tong *et al*[7]	17 (14 case-control, 3 cohort)	14667 pancreatic cancer, 17587 pancreatitis	Pooled OR, 10.35 (95%CI: 9.13-11.75) for chronic pancreatitis; Pooled OR, 6.41 (95%CI: 4.93-8.34) for unspecified type of pancreatitis -	Inclusion of studies with both acute and chronic pancreatitis limits generalizability; Inclusion of acute, chronic and unspecified pancreatitis to calculate the temporality of the association might underestimate the relative risk
Kirkegård *et al*[8]	13 (4 case-control, 9 cohort)	674 pancreatic cancer, 25,329 chronic pancreatitis	EE, 16.16 (95%CI:12.59-20.73) for 2-yr lag period; EE, 7.90 (95%CI: 4.26-14.66) for 5-yr lag period; EE, 3.53 (95%CI: 1.69-7.38) for 9-yr lag period	Some of the included studies had a smaller number of patients with pancreatic cancer or chronic pancreatitis which might inflate the estimated risk; In some of the included studies more than one-third of patients were lost to follow-up

CI: Confidence interval; EE: Effect estimate; OR: Odds ratio; RR: Relative risk.

Table 2 Risk factors for pancreatic cancer in chronic pancreatitis

Ref.	Number of patients	Etiological subtype, *n* (%)	Pancreatic cancer risk	Specific risk group
Agarwal *et al*[22]	1415	ACP, 540 (38.1); IJCP, 668 (47.2); ISCP, 207 (14.6)	5-yr risk of pancreatic cancer: ACP, 0.5%; IJCP, 0.2%; ISCP, 3.7%. 10-yr risk of pancreatic cancer: ACP, 0.9%; IJCP, 0.2%; ISCP, 5.2%	Age of onset > 35 yr (HR 12.1; CI: 4.7-31.2; $P < 0.001$). Smoking (HR 6.48; CI: 2.2-19.0; $P < 0.001$). Nonalcoholic etiology (HR for alcohol use 0.14; CI: 0.04-0.42; $P < 0.001$); Absence of steatorrhea (HR for steatorrhea 0.19; 95%CI: 0.04-0.80; $P < 0.023$)
Hao *et al*[23]	2037	ACP, 404 (19.8); ICP, 1633 (80.2)	5-yr risk of pancreatic cancer: ACP, 0.2%; ICP, 0.7%. 10-yr risk of pancreatic cancer: ACP, 0.9%; ICP, 1.1%	No significant difference in pancreatic cancer development between ACP and ICP patients
Vujasinovic *et al*[25]	581	Idiopathic, 47 (8.1); Alcohol and nicotine, 220 (37.9); Nicotine, 81 (13.9); Alcohol, 49 (8.4); Hereditary, 38 (6.5) Immunological, 59 (10.2) Efferent duct 62 (10.7) Miscellaneous/other, 25 (4.3)	Rate per 100 yr; Idiopathic, 0.00; Alcohol and nicotine, 0.07; Nicotine, 0.44; Alcohol, 0.32; Hereditary, 0.00; Immunological, 0.00; Efferent duct, 0.28; Miscellaneous/other, 0.81	Low BMI and pancreatic exocrine insufficiency. Patients with diabetes mellitus and high BMI
Müllhaupt *et al*[24]	332	ACP, 265; IJCP, 21; ISCP, 46	ACP, 1.5% over 16 yr; IJCP, 4.8% over 22 yr; ISCP, 2.2% over 14 yr	Early onset and prolonged duration of CP (rather than the specific type of CP)

ACP: Alcoholic pancreatitis; BMI: Body mass index; ICP: Idiopathic chronic pancreatitis; IJCP: Idiopathic juvenile chronic pancreatitis; ISCP: Idiopathic senile chronic pancreatitis.

(Figure 1). Of the various predisposing factors, smoking and alcohol intake are the important modifiable risk factors relevant to pancreatic carcinogenesis in a genetically susceptible individual. The genes implicated in the pathogenesis of pancreatitis are protease serine 1 (*PRSS1*), serine peptidase inhibitor Kazal type 1 (*SPINK 1*), Cystic fibrosis transmembrane conductance regulator (*CFTR*), chymotrypsin C (*CTRC*) and calcium-sensing receptor (*CASC*)[28-30]. Recently mutations in the genes encoding carboxypeptidase A1 (CPA1), carboxyl ester lipase, and tight-junction protein claudin-2 (CLDN2) have also been postulated to initiate pancreatitis[29]. Gain-of-function mutations in the *PRSS1* gene, which encodes cationic trypsinogen, facilitate intrapancreatic trypsin activation[31]. Mutation of the *SPINK 1* gene that codes for trypsin inhibitor, together with CTRC and CASC mutations, promotes abnormal trypsin activation[32]. While PRSS1, SPINK1, CTRC and CASC initiate pancreatitis through a trypsin-dependent pathway, CFTR mutation affects the anion channel for chloride and bicarbonate[30]. Disturbed alkalinization of the pancreatic juice results in injury to the duct epithelium followed by protein plug and pancreatic stone formation[29,33]. In addition to the initiation of pancreatic inflammation, these genetic factors play an

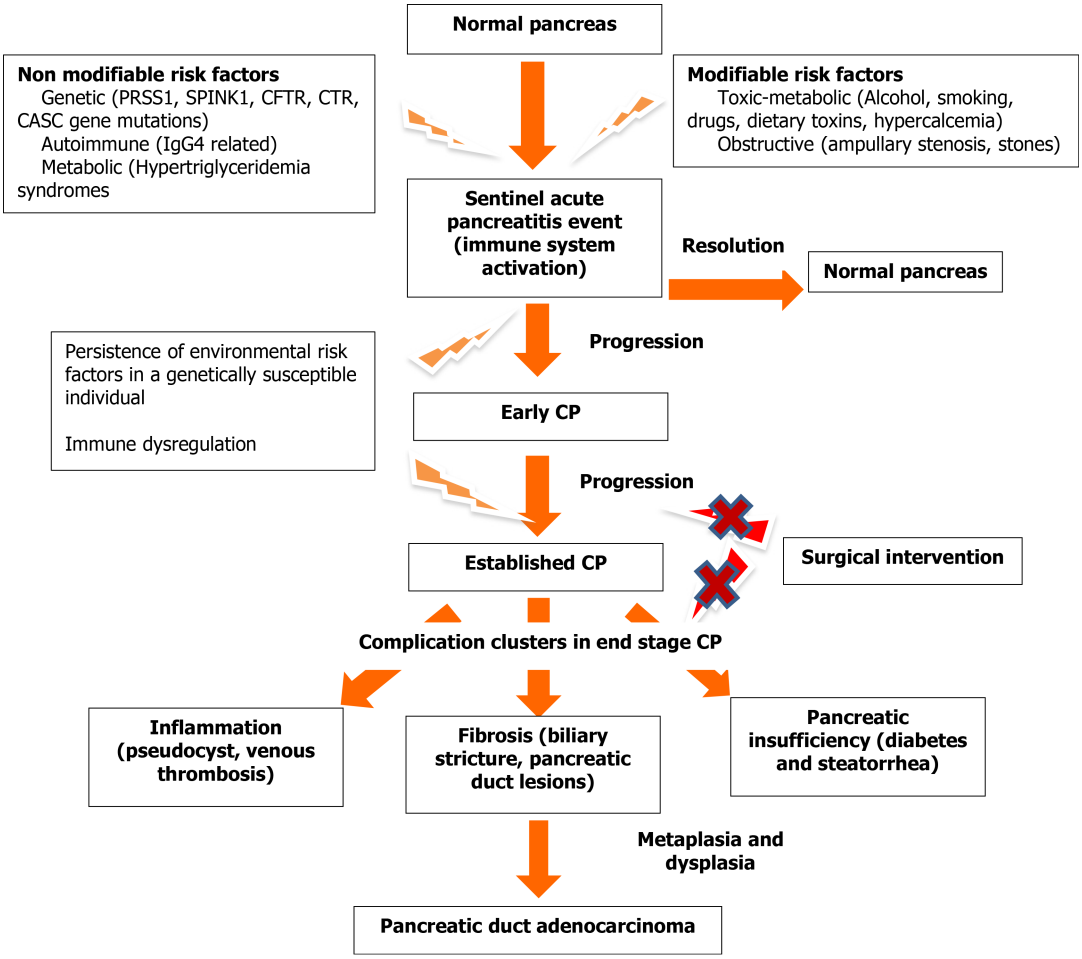


Figure 1 Pathogenesis of chronic pancreatitis and chronic pancreatitis-related pancreatic cancer. *CASC*: Calcium-sensing receptor gene; *CFTR*: Cystic fibrosis transmembrane conductance regulator gene; CP: Chronic pancreatitis; *CTR*: Chymotrypsin C gene; *PRSS1*: Protease serine 1 gene; *SPINK 1*: Serine peptidase inhibitor Kazal type 1 gene.

important role in the progression of acute inflammation to CP[30]. Recent studies have shown that the clinical manifestations of CP can be grouped into three complication clusters[34]. One group of patients has dominant inflammatory complications like pseudocyst, pancreatic ascites, and vascular (splenic or portal vein) thrombosis. The second group has predominant pancreatic exocrine (steatorrhea) and endocrine (diabetes) insufficiency. The third group has fibrotic complications characterized by biliary stricture, duodenal stenosis, and pancreatic duct lesions. Individual patients can have more than one complication cluster, but most have a single dominant complication cluster. As patients with predominant fibrotic complications are at increased risk of PC, understanding the inflammatory and gene pathways can help in the risk stratification of individual patients[22,34].

It is well known that PC tissue includes malignant duct cells surrounded by stroma that accounts for more than 90% of the tumor mass[35]. The histology of CP tissue includes fibrotic destruction of the pancreatic parenchyma, infiltration of inflammatory cells, and ductal and exocrine atrophy[2]. As pancreatic stellate cells are the principal source of fibrosis, their activation has been implicated in the pathogenesis of CP and CP-related PC[36-38] (Figure 2). Activation of the immune system following injury of the pancreatic duct and acini results in the release of multiple proinflammatory cytokines including tumor necrosis factor (TNF)-α and interleukin (IL)-1, IL-6, and IL-8, growth factors, chemokines including monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1, adhesion molecules, reactive oxygen and reactive nitrogen species that activate pancreatic stellate cells and induce proliferation[39,40]. Transforming growth factor (TGF) β1 and fibroblast growth factor produced by pancreatic stellate cells stimulate extracellular matrix synthesis and cyclooxygenase-2 mediates extracellular matrix proliferation[36-38]. The benign process of inflammation-related proliferation and fibrosis can turn malignant in the background of uncontrolled inflammation. This is supported by the observation that

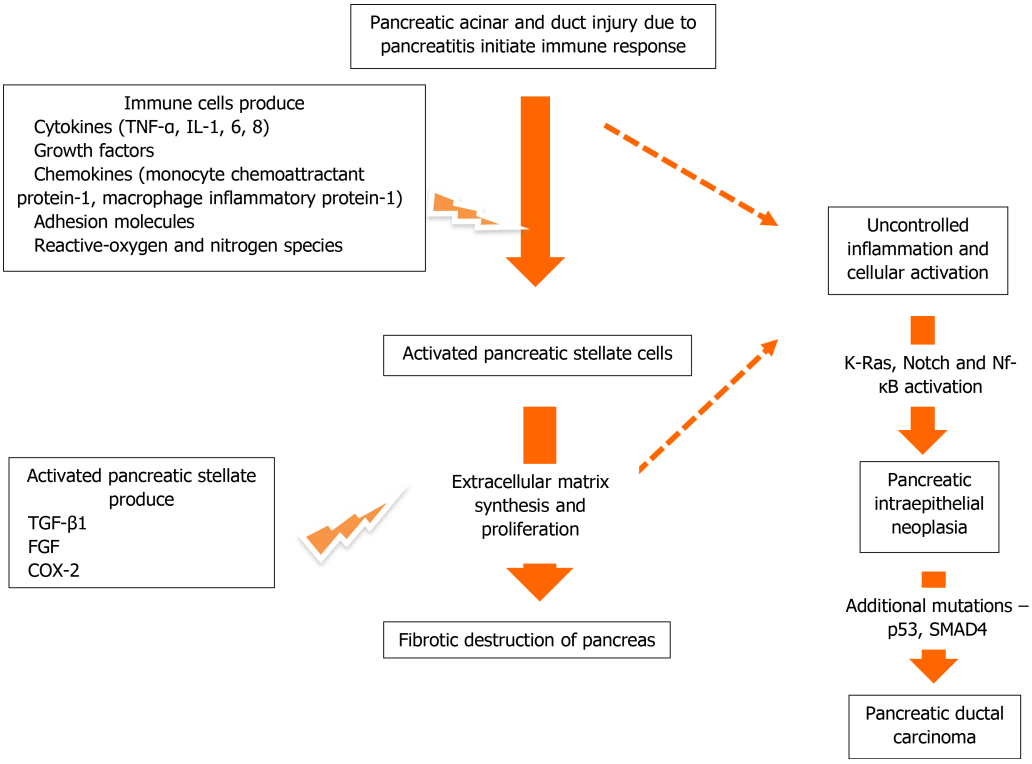


Figure 2 Mechanisms linking chronic pancreatitis and pancreatic cancer. COX-2: Cyclooxygenase-2; FGF: Fibroblast growth factor; IL: Interleukin. TGF-β1: Transforming growth factor β1; TNF: Tumor necrosis factor.

mutation of the *K-Ras* proto-oncogene stimulates tumor development only in the presence of inflammatory milieu; inflammatory mediators are required for its activation[41-44]. Approximately 30% of CP patients with CP have K-Ras mutations [44]. K-Ras mutation itself is not sufficient, the pathological activity necessary for PC development is achieved only in the presence of persistent inflammation[41]. Additionally, activation of the Notch signaling pathway and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factor promotes carcinogenesis against an inflammatory background[45]. The current understanding of the inflammatory and oncogene pathways of PC in CP patients is that CP significantly increases the risk of PC (relative risk > 2) compared with other risk factors, and that inflammation plays a crucial role in carcinogenesis[20].

Genetic mutations responsible for hereditary pancreatitis are not directly associated with the development of PC. However, genetic susceptibility increases the possibility of recurrent and persistent inflammation, especially in the presence of environmental risk factors like smoking and alcohol intake, thereby increasing the risk of PC[46]. Among the genetic causes of pancreatitis, *PRSS1* mutation carries the maximum risk for PC followed by other mutations. The difference in risk primarily results from the ability to initiate and sustain an inflammatory cascade with or without other environmental factors[47]. Differences in the risk of PC in IJCP in Indian and European studies are caused by differences in the underlying genetic mutations. IJCP is predominantly associated with CFTR and SPINK 1 mutations in the Indian population and with PRSS1 mutation in European populations[48].

Prospective cohort studies of patients with hereditary pancreatitis have reported that avoidance of smoking and alcohol intake, ensuring a smoke-free environment in the home and workplace, maintaining normal BMI, and eating a healthy diet reduces the cumulative risk of PC[20,50]. The success of cancer preventive measures suggests that medical, endoscopic, and surgical interventions to minimize ongoing pancreatic inflammation can reduce the risk of PC. Alcohol consumption increases the risk of pancreatitis and smoking increases the risk of fibrotic complications in CP and related PC. Experimental studies have shown that aryl hydrocarbon receptor ligands in cigarette smoke induce production of interleukin-22 and pancreatic fibrosis. Hence, lifestyle modifications like smoking cessation can minimize the risk of PC[51]. Risk stratification of CP patients is proposed based on the current understanding of the inflammatory and oncogene pathways of CP-induced PC (Figure 3). The proposed risk stratification underlines the need for genetic testing in most patients with CP, as the

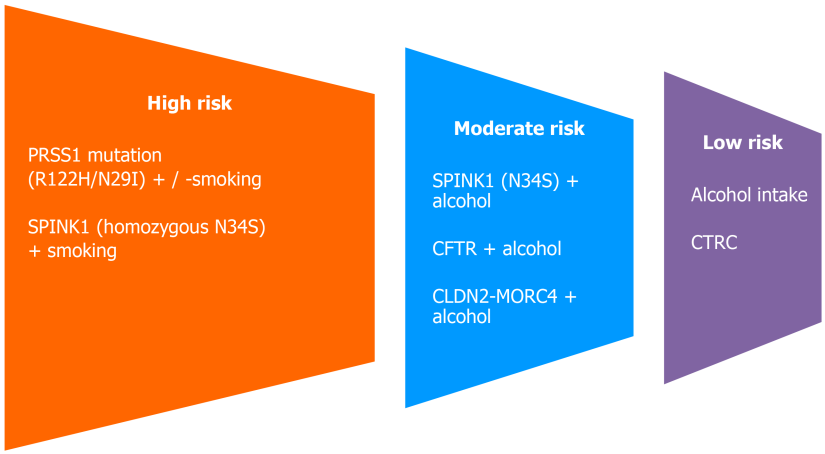


Figure 3 Proposed risk stratification of chronic pancreatitis patients. CFTR: Cystic fibrosis transmembrane conductance regulator; CLDN2: Tight-junction protein claudin-2; CTRC: Chymotrypsin C; PRSS 1: Protease serine 1; SPINK 1: Serine peptidase inhibitor Kazal type 1.

combination of genetic and environmental factors determines the risk of PC.

DOES SURGERY FOR CP REDUCE THE RISK OF PC?

Although CP is considered a risk factor for PC, the impact of surgery for CP on the risk of PC has not been well studied. To investigate the relationship between surgery for CP and PC, Ueda *et al*[12] performed a multicenter retrospective analysis of 893 CP patients at 22 institutions who fulfilled the 2001 Japan Pancreas Society diagnostic criteria for CP. They excluded 387 patients with less than 2 years of follow-up or a lag period of less than 2 years between CP diagnosis and PC. Of the 506 patients included in the study, 19 (3.7%) developed PC (Table 3). The standardized incidence ratio (SIR), defined as the ratio of observed to expected PC cases, was 11.8 (95%CI, 7.1-18.4). The cumulative incidence (95%CI) of PC was 2.0% (1.0-4.1) at 5; 4.6% (2.6-8.2) at 10; 7.5% (4.3-12.9) at 15; 10.7% (4.3-18.4) at 20, and 14.0% (7.5%-25.3%) at 25 years after the CP diagnosis. At a median follow-up of more than 5 years, the PC incidence was significantly lower in patients who had undergone surgery (1/147, 0.7%0) than in those who did not undergo surgical intervention (18/352, 5.1%) for CP (P = 0.03]. Based on the results, the authors concluded that surgery for CP decreased the risk of PC[12].

Zheng *et al*[11] performed a retrospective analysis of 650 surgically managed CP patients to determine the incidence and risk factors for PC after surgery for CP. At a median follow-up of 4.4 years, 12 patients (1.8%) developed PC (Table 3). The SIR (95%CI) for PC in CP patients was 68.12 (35.20-118.99). The cumulative incidence (95%CI) of PC was 1.48% (0.46-2.51) at 3; 2.63% (0.93-4.32) at 6; and 3.71% (1.05-6.37%) at 9 years after CP surgery. The time interval to surgery, defined as the lag time between the initial CP diagnosis and operative intervention, was significantly longer in CP patients who developed PC (96.6 ± 164.4 mo *vs* 36.4 ± 74.2 mo, P = 0.016), with a hazard ratio (95%CI) of 1.005 (1.002-1.008, P = 0.002). Based on the results, the authors concluded that early surgical intervention for CP prevented the development of CP-related PC[11].

The proposed mechanisms by which surgery for CP reduces PC incidence are relief of pancreatic compartment syndrome and inflammation by resection of fibrotic parenchyma and surgical drainage[11]. As mentioned earlier, parenchymal fibrosis plays an essential role in pancreatic carcinogenesis, and its removal reduces the volume of pancreatic tissue at risk of malignancy. This hypothesis is supported by studies in which none of the patients who underwent pancreatoduodenectomy for CP developed PC during the follow-up period[11,12]. As persistent uncontrolled inflammation is a significant risk factor for developing PC, relief of ductal obstruction and pancreatic compartment syndrome attenuates pancreatic inflammation.

Table 3 Studies evaluating the impact of surgery for chronic pancreatitis on the risk of pancreatic cancer

Ref.	Number of patients	Etiological subtype, *n* (%)	Type of surgery, *n* (%)	Pancreatic cancer risk
Ueda *et al*[12]	506	Alcoholic pancreatitis, 345 (68.0); Idiopathic pancreatitis, 109 (22.0); Autoimmune pancreatitis, 16 (3); Hereditary pancreatitis, 9 (2); Miscellaneous, 27 (5)	Drainage operation, 60 (40.8); Pancreatic resection, 87 (59.2)	Surgery, 1/147 (0.7%); No surgery, 18/352 (5.1%)
Zheng *et al*[11]	650	Alcoholic pancreatitis, 361 (55.5); Idiopathic pancreatitis, 227 (34.9); Hereditary pancreatitis, 62 (9.5)	Drainage operation, 189 (29.1); Pancreatic resection, 461 (70.9)	Surgery, 12/650 (1.8%); Time interval to surgery, HR, 1.005 (95%CI: 1.002-1.008); *De novo* endocrine insufficiency, HR, 10.672 (95%CI: 2.567-44.372)

IS PC RISK REDUCTION AN INDICATION FOR SURGERY IN CP PATIENTS?

The standard indications for surgery in CP are intractable pain, suspicion of malignancy, and local pancreatic or extrapancreatic complications, such as biliary stricture, duodenal stenosis, pseudocysts, and vascular complications (*e.g.*, splenic/portal vein thrombosis and arterial pseudoaneurysms)[2,4], and intractable pain is the most common indication. Traditionally, patients are managed by a step-up approach that begins with medical management (analgesics) followed by endoscopic intervention[52,53]. Surgery is generally considered as the last option after multiple endoscopic interventions fail to provide adequate symptomatic relief. However, based on the findings of numerous observational studies and a recent randomized controlled trial, international consensus guidelines for surgery and the timing of intervention in CP recommend early surgery[4]. Early surgery, *i.e.* performed within 3 years of the onset of CP symptoms, is associated with better pain relief and preservation of pancreatic function[3,54]. Considering recent evidence for early surgery in CP and its potential protective role in the development of PC, the clinician faces the question of prophylactic surgery in CP patients. Prophylactic surgery for CP is performed with the primary aim of reducing the risk of PC in the absence of typical surgical indications like pain and local complications. The dilemma is significant in light of the current availability of digitized clinical information for patients and increased litigation. Is the clinician is ethically right or legally justified in not offering a treatment that can prevent a deadly complication of CP? To answer that question, a critical analysis of the current evidence is essential. Many studies have evaluated the risk of PC, but only a few have investigated the protective effect of surgery for CP on reducing the risk of PC [11,12]. Limitations of previous studies are the inclusion of small numbers of patients and retrospective analysis, which make the accuracy and completeness of the study data difficult to control. Confounding risk factors like smoking and alcohol intake were not accurately quantified in most studies, and the proportion of patients who underwent resection procedures was relatively high, in at least in one of the studies, which contrasts with the drainage procedures commonly performed worldwide for CP patients[55]. Another limitation is the lack of objective evidence to document whether pancreatic inflammation was reduced after surgery. As persistent pancreatic inflammation is considered a significant risk factor for pancreatic carcinogenesis, a reliable biomarker to monitor the presence or absence of persistent pancreatic inflammation after surgery could help us better understand the role of surgery in reducing the risk of PC. Hence, to confirm the protective role of surgical procedures for CP, additional prospective studies involving large populations are required. While the risk of PC is increased in patients with CP, it is still too low to recommend routine active screening or prophylactic surgery in all patients[20]. With the available evidence, routine surveillance and prophylactic surgery are recommended only in subgroups of hereditary pancreatitis with a high risk of PC. In contrast to environmental risk factors, genetic factors are not modifiable. Hence, prophylactic surgery will have a greater role in this group of patients. Until we have more evidence, surgery for CP is currently recommended for only the described standard indications.

FUTURE DIRECTIONS: PRECISION MEDICINE IN CP

The mechanistic definition of CP is a "pathologic fibroinflammatory syndrome of the pancreas in individuals with genetic, environmental and/or other risk factors who

develop persistent pathologic responses to parenchymal injury or stress"[13]. The current definition highlights the disease heterogeneity in terms of individual susceptibility, disease onset, modifying factors, rate of progression, and long-term outcomes. Detailed evaluation of an individual patient often identifies a genetic factor that predisposes to pancreatitis in the presence of environmental risk factors[29,30]. The complex interaction of genetic and environmental factors determines the clinical phenotype and risk of PC. Hence, the concept of precision medicine used in various complex benign and malignant gastrointestinal disorders should be employed in CP patients. Molecular pathways leading to CP-related PC should be better defined so that complication clusters in an individual patient and the role of interventions in mitigating the cancer risk can be well studied.

Advances in instrumentation and technology allow increasing use of endoscopic interventions in CP patients. However, long-term studies focusing on the risk of PC in patients undergoing endoscopic intervention are lacking. As previously mentioned, reliable markers for pancreatic inflammation would help to evaluate the success of endoscopic and surgical treatment in individual patients. Hence future prospective studies evaluating the role of surgical and endoscopic intervention in reducing PC should include accurate risk stratification of the study patients and preferably incorporate biomarkers of pancreatic inflammation.

CONCLUSION

CP is a significant risk factor for PC. However, the relative risk is as not high as previously reported. Persistent pancreatic inflammation plays a key role in pancreatic carcinogenesis, and genetic mutations implicated in the pathogenesis of CP are not directly involved in the development of PC. Activation of pancreatic stellate cells and enhanced K-Ras oncogene activity in the inflammatory milieu play a key role in the pathogenesis of CP-related PC. Although surgery for CP reduces the risk of PC, the evidence is not strong enough to recommend routine prophylactic surgery in CP patients. The proposed risk stratification considers the genetic and environmental factors that could guide future prospective studies and select patients for prophylactic surgery.

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MINIREVIEWS

Neoadjuvant therapy for pancreatic ductal adenocarcinoma: Opportunities for personalized cancer care

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy that is best treated in a multidisciplinary fashion using surgery, chemotherapy, and radiation. Adjuvant chemotherapy has shown to have a significant survival benefit in patients with resected PDAC. However, up to 50% of patients fail to receive adjuvant chemotherapy due to postoperative complications, poor patient performance status or early disease progression. In order to ensure the delivery of chemotherapy, an alternative strategy is to administer systemic treatment prior to surgery. Precision oncology refers to the application of diverse strategies to target therapies specific to characteristics of a patient's cancer. While traditionally emphasized in selecting targeted therapies based on molecular, genetic, and radiographic biomarkers for patients with metastatic disease, the neoadjuvant setting is a prime opportunity to utilize personalized approaches. In this article, we describe the current evidence for the use of neoadjuvant therapy (NT) and highlight unique opportunities for personalized care in patients with PDAC undergoing NT.

Key Words: Pancreatic cancer; Preoperative therapy; FOLFIRINOX; Precision oncology; Patient-reported outcomes; Personalized medicine; Targeted therapy; Whipple; Pancreatectomy

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Core Tip: Neoadjuvant therapy (NT) is an increasingly utilized approach that maximizes the receipt of multimodality therapy, improves margin-negative resection rates, and potentially increases survival durations. In the era of personalized medicine, the neoadjuvant period can also be used to emphasize precision oncology. Already,

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current methods of anatomically staging, molecularly profiling, and monitoring response to therapy can be used to personalize neoadjuvant treatment for localized pancreatic ductal adenocarcinoma (PDAC). In this article, we describe the current evidence for the use of NT and highlight unique opportunities for personalized care in patients with PDAC undergoing NT.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the leading causes of cancer-related deaths worldwide with a 5-year overall survival (OS) rate of only 10%[1]. Despite surgical resection being the only hope for cure, only a small proportion of patients present with resectable disease and the majority of patients will develop locoregional or metastatic recurrence after surgery[2,3]. Adjuvant chemotherapy has shown to have a significant survival benefit in patients with resected PDAC[3]. However, up to 50% of patients fail to receive adjuvant chemotherapy due to postoperative complications, poor patient performance status or early disease progression[4-6].

In order to ensure the delivery of chemotherapy, an alternative strategy is to administer systemic treatment prior to surgery. Neoadjuvant therapy (NT) has been shown to confer several clinical benefits such as improved margin-negative resection rates, decreased lymph node positivity, early treatment of presumed micro-metastatic disease, an optimal window of time to provide prehabilitation before surgery, and the ability to measure *in vivo* response to therapy histologically after resection[7-9]. Moreover, increasing evidence from randomized controlled trials (RCT) suggests NT may improve OS in patients with non-metastatic PDAC compared with upfront resection[10,11]. Despite the advantages of NT, its use in the United States has remained relatively low[12,13]. While NT is now the recommended treatment strategy for borderline resectable (BR) or locally advanced cancer, current NCCN guidelines support either upfront surgical resection or NT for patients with resectable disease[14].

There is growing interest in emphasizing personalized approaches to multidisciplinary cancer care that reflects not only unique differences in cancer biology but also individual circumstances and treatment goals. Precision oncology refers to the application of diverse strategies to target therapies specific to characteristics of a patient's cancer. While traditionally emphasized in selecting targeted therapies based on molecular, genetic, and radiographic biomarkers for patients with metastatic disease, the neoadjuvant setting is a prime opportunity to utilize personalized approaches. In this article, we describe the current evidence for the use of NT and highlight unique opportunities for personalized care in patients with PDAC undergoing NT.

RATIONALE FOR NT

Large cohort and population-based studies have shown that as many as 50% of patients who undergo surgical resection for PDAC are unable to receive adjuvant therapy due to postoperative complications, poor performance status, or early disease progression[4-6,15,16]. Even among healthy patients enrolled in clinical trials, a substantial proportion of patients are unable to initiate adjuvant therapy due to the morbidity of pancreatic surgery[4]. An even greater proportion of patients fail to complete all intended cycles of adjuvant therapy[5]. Thus, the ability to ensure receipt of systemic therapy and facilitate multimodality therapy is one of the strongest reasons to recommend NT. Other advantages that support the use of NT include the early treatment of presumed micrometastatic disease, the ability to potentially downstage BR disease improving the chances of R0 resection, and improved patient selection by

avoiding surgery in those patients with rapid disease progression during preoperative treatment. Additionally, well-oxygenated, non-devascularized tissue is more susceptible to the effects of chemoradiation, which theoretically increases the efficacy of chemoradiation if given prior to surgery[17,18].

These advantages must be carefully weighed against the potential disadvantages of pursuing NT. First, unlike in a surgery-first approach, tissue diagnosis and biliary decompression are uniformly required. These procedures may delay the initiation of treatment and are associated with small, but non-zero, risks. Second, NT is inherently multi-disciplinary and require careful coordination among providers. Third, and most importantly, delivering aggressive chemotherapy and/or radiation prior to surgery can lead to severe toxicity that, in extreme cases, can preclude subsequent surgical resection. Indeed, a systematic review by the Dutch Pancreatic Cancer Group calculated a Grade III or higher toxicity rate of 64% among patients undergoing NT [19]. The recent SWOG S1505 trial of NT for resectable PDAC found that nearly 13% that started NT were unable to undergo surgery because of performance status decline [20]. Finally, while distant progression while on NT is far more common, a small risk of local progression that leads to unresectability exists. These challenges highlight the importance not only of personalizing treatment decisions regarding NT, but also of emphasizing research that improves the delivery of NT for patients with localized PDAC.

CURRENT EVIDENCE FOR NT

Support for the use of NT for localized PDAC has largely come from small prospective trials, single-institutional series, and cancer registries[21]. These studies largely demonstrated its feasibility and proposed NT as an acceptable approach for PDAC. Suggestions of improved outcomes compared to upfront surgery have been limited by study design. However, the completion of several RCT in recent years comparing NT to upfront surgery have generated increased support for NT. For example, two RCTs found improved margin-negative resection rates and OS among patients with BR PDAC[22] who received neoadjuvant CRT compared to immediate surgery. Furthermore, the PACT-15 and Prep-02/JSAP-05 RCTs found improved OS among patients with resectable PDAC treated with neoadjuvant chemotherapy compared to those who underwent immediate surgery[16,23].

In a recent meta-analysis of only prospective RCTs, Cloyd *et al*[13] showed that the OS of patients with resectable or BR PDAC who received NT was nearly 30% better than that of patients who underwent surgery upfront using an intention-to-treat design. Furthermore, the meta-analysis found that NT improved R0 resection rate and decreased lymph node positivity rate. Since then, preliminary results from the ESPAC-5 trial, a four-arm RCT comparing patients undergoing surgery upfront, neoadjuvant gemcitabine/capecitabine (GEMCAP), neoadjuvant FOLFIRINOX, and neoadjuvant capecitabine-based radiation, showed improved one-year OS among patients receiving NT[24]. As a limitation of previous RCTs is the use of non-traditional neoadjuvant regimens (*e.g.*, folfirinox or gemcitabine-abraxane), the long-term results of this trial and other contemporary studies comparing NT to immediate surgery are anxiously awaited.

OPPORTUNITIES FOR PERSONALIZED CANCER CARE

Several factors make the neoadjuvant period an optimal scenario to emphasize precision oncology (Table 1). First, PDAC is anatomically and genetically heterogeneous as is the clinical presentation of patients with localized disease. Second, patient and tumor response to NT differs significantly. Evaluating and responding to this dynamic staging offers an opportunity to personalize subsequent treatment.

Anatomic staging

Using high-quality cross-sectional imaging, localized PDAC is classified as resectable, BR or locally advanced (LA; also termed unresectable) according to its relationship with major vascular structures. While several organizations have published staging criteria with only slight differences, the SSO/SSAT/AHPBA consensus definitions are commonly employed. As these criteria reflect the likelihood of achieving a margin-negative resection with upfront surgery, the development of a uniform anatomic

Table 1 Opportunities for personalized care during neoadjuvant therapy for pancreatic cancer

Concept	Description	Examples	Outcome
Anatomic staging	Characterization of local extent and vascular involvement of tumor	Locally advanced/unresectable; Borderline resectable; Potentially resectable	Anatomic staging can influence the recommended duration and components (*e.g.*, preoperative radiation) of neoadjuvant therapy
Molecular staging	Identification of tumor/germline genetic and molecular markers	*BRCA* mutations; Mismatch repair-deficiency; Molecular markers	Specific tumor/germline mutations may identify opportunity for targeted therapies (*e.g.*, immunotherapy, PARP inhibitors) Standard chemotherapy may be influenced by molecular markers (*e.g.*, resistance/sensitivity to traditional flouropyridamine or gemcitabine-based therapy)
Dynamic staging	Measuring biochemical, radiographic, and histologic response of the tumor to neoadjuvant therapy	Carbohydrate antigen 19-9; Response evaluation criteria in solid tumors response; Pathologic response	Measuring response to neoadjuvant therapy can influence treatment strategies (*e.g.*, changing neoadjuvant regimen, use of radiation, recommendations for adjuvant therapy)

staging system has been pivotal for improved clinical protocol standardization specifically concerning the use of NT[25] (Table 2).

LA PDAC truly represents unresectable disease, typically because of arterial encasement or non-reconstructable venous involvement. Still, recent studies have found that a small but significant proportion of patients can be converted to resectable disease after aggressive NT[26]. For example, a large retrospective study by Hackert *et al*[27] showed that 61% of patients with LA PADC receiving FOLFIRINOX as NT underwent successful surgical resection with a 40.8% R0 resection rate. Another study by Gemenetzis *et al*[28] showed that FOLFIRINOX-based therapy and stereotactic body radiation therapy correlated with increased probability of resection ($P = 0.006$); patients who eventually underwent surgical resection had higher median OS compared with those who did not (35.3 mo *vs* 16.3 mo; $P < 0.0001$). Given the vascular involvement and low likelihood of achieving negative microscopic margins, preoperative radiation therapy is commonly employed after induction systemic chemotherapy prior to attempts at surgical resection. This approach is logical since consolidative radiation is frequently administered for patients with LA disease who are not surgical candidates to enhance locoregional control[29].

A similar approach should be considered for patients with BR PDAC yet with higher likelihood of undergoing surgical resection. Current practice is to typically begin with induction systemic chemotherapy. For example, the Alliance for Clinical Trials in Oncology group A021101, demonstrated a 93% R0 resection rate for patients with BR PDAC after receiving FOLFIRINOX followed by capecitabine-based chemoradiation as NT[30]. Similarly, a single-arm prospective trial of neoadjuvant FOLFIRINOX followed by radiation resulted in a high degree of R0 resection in those who underwent surgery with impressive progression-free survival (PFS) and OS durations [31]. Despite these results, the role of radiation following induction chemotherapy remains controversial for BR PDAC. The Alliance A021501 trial, randomized patients with BR PDAC to either 8 cycles of FOLFIRINOX or 7 cycles of FOLFIRINOX followed by hypofractionated radiation before surgery. Patients who received radiation therapy were less likely to undergo surgical resection and experienced shorter OS[32]. In summary, NT with systemic chemotherapy with selective use of preoperative radiation is currently the preferred approach for BR PDAC.

The use of NT remains the most controversial in patients with resectable PDAC and wide variation in practices exist[21]. Nevertheless, the use of NT continues to increase given the previously described rationale and increasing evidence that highlights improved outcomes. However, given the lesser need for downstaging and higher likelihood of achieving an R0 resection, current neoadjuvant approaches tend to utilize systemic chemotherapy alone. For example, the SWOG S1505 trial was a recent RCT of either neoadjuvant mFOLFIRINOX or gemcitabine/nab-paclitaxel prior to surgical resection for resectable PDAC as NT[33]. In addition, the recently opened Alliance A021806 will randomize patients with resectable PDAC to either perioperative mFOLFIRINOX or surgery upfront followed by adjuvant mFOLFIRINOX[34].

Molecular staging

The last decade has seen dramatic advances in our understanding of the genetic underpinnings of PDAC pathogenesis. With advanced tumor profiling and next-generation sequencing, recent studies have characterized the frequency of genetic and molecular alterations in PDAC tumors[35]. This information, if obtained routinely *via* tissue biopsy or surgical specimen, can contribute to a more personalized approach to

Table 2 Anatomic staging of localized pancreatic ductal adenocarcinoma as defined by the 2008 AHPBA/SSO/SSAT consensus guidelines

	Resectable	Borderline	Locally advanced
SMV-PV	Uninvolved with tumor with clear fat planes around vessels	Abutment, encasement, or occlusion of short segment of vein	Occlusion, thrombosis, or encasement extending several centimeters
SMA	Uninvolved	Tumor abutment < 180°	Tumor abutment > 180° (encasement) or thrombosis of artery
Celiac axis	Uninvolved	Uninvolved celiac axis; short segment encasement or abutment of common hepatic artery may be amenable to resection and reconstruction	Abutment or encasement of celiac axis indicates unresectability

SMV: Superior mesenteric vein; PV: Portal vein; SMA: Superior mesenteric artery.

treatment *via* molecular profiling[36]. In a study by Krepline *et al*[37], 73 out of 78 resected PDAC specimens were found to have a pathogenic variant on NGS of which 18% were potentially actionable. For example, patients with BRCA1/BRCA2 mutations are known to benefit from platinum-based chemotherapy and PARP inhibitors[38,39]. Pancreatic cancer that is mismatch repair-deficient (dMMR), a small but clinically relevant proportion, are known to respond to immunotherapy[40,41]. Finally, the efficacy of standard chemotherapy regimens can be modified depending on thymidylate synthase (TYMS), excision repair cross-complementing (ERCC1) protein, ribonucleotide reductase M1 (RMM1), secreted protein acid and rich in cysteine (SPARC), topoisomerase I (TOP1), and human equilibrative nucleoside transported 1 (hENT1) levels. Low TYMS, ERCC1 protein, and RRM1 Levels predict efficacy of 5-FU based therapies and capecitabine, cisplatin, and gemcitabine respectively[42-44]. Elevated SPARC level predicted sensitivity to nab-paclitaxel while low levels of hENT1 have been associated with gemcitabine resistance[43,45].

These molecular biomarkers, if obtained from preoperative endoscopic ultrasound biopsies, can thus be used to tailor NT. In a recent prospective trial, Tsai *et al*[46] delivered neoadjuvant systemic therapy to 130 patients based on the molecular profile results. In this trial, 6 molecular targets were utilized to predict chemosensitivity: TYMS, ERCC1, RMM1, SPARC, TOP1, and hENT1. Of the 92 patients with predictive molecular profiling, 74 (80%) received fluoropyrimidine-based systemic therapy and 18 (20%) received gemcitabine-based systemic therapy[46]. Of the 130 overall patients enrolled, 107 (82%) completed all intended NT followed by surgical resection including 56 (92%) with resectable PDAC and 51 (74%) with BR PDAC. The importance of this trial was to demonstrate the increased rate of resectability, which suggests that molecular profiling potentially improved the efficacy of NT[46]. Furthermore, the Individualized Molecular Pancreatic Cancer Therapy (IMPaCT) trial documented the feasibility of acquiring and screening pancreatic tumor tissue for HER2 amplification, KRAS mutation, and mutations in BRCA1, BRCA2, PALB2, and ATM[47]. A pilot study using molecular profiling in a wide variety of metastatic cancers demonstrated a longer PFS in 27% of patients receiving molecular profiling-based systemic therapy[36]. The scarcity of studies in this field suggest that this method of personalized care is under-utilized and warrants further investigation.

Dynamic staging

In contrast to standard adjuvant therapy, administering nonoperative therapies prior to surgery provides a unique opportunity to measure the tumor response to treatment *in vivo*. Traditional measures of tumor response to NT include biochemical, radiographic, and histologic. There is abundant data supporting the use of carbohydrate antigen 19-9 (CA 19-9) in the clinical management of patients with PDAC. While baseline CA 19-9 is an important measure of disease burden, numerous studies have highlighted the prognostic significance of CA 19-9 response to NT[48-52]. For example, Boone *et al*[49] found that CA 19-9 response to NT was associated with R0 resection rate, histopathological response, and OS.

Restaging with cross-sectional imaging is routinely performed during and following NT, mostly to rule out disease progression but also to assess the response of the primary tumor to treatment. While the Response Evaluation Criteria in Solid Tumors (RECIST) grading system has historically felt to under-represent treatment response [53], Perri *et al*[54] recently found in a large retrospective study that RECIST partial response and a reduction in tumor volume after NT were independently associated

with pathologic response in patients with localized PDAC. In addition to serologic and radiographic measures, the response to NT can be measured histologically in surgical specimens as the proportion of active cancer cells. Unfortunately, previous studies have found that only a major pathologic response (defined as < 5% active cancer cells) is associated with improved prognosis[8] and that pathologic complete responses are rare[55].

A dynamic assessment of tumor response to treatment may help personalize treatment in several ways. First, non-responders to induction systemic chemotherapy can be switched to alternative regimens. In a study by Vreeland *et al*[56], of 25 BR or LA PDAC patients who did not respond to FOLFIRINOX after 4 mo of treatment as NT, 21 (84%) showed a serologic or radiographic response after switching to gemcitabine/nab-paclitaxel and 11 of them underwent surgical resection (Figure 1). In contrast, responders who are tolerating therapy may be selected to continue this regimen particularly if CA 19-9 has not normalized yet or additional downstaging is required. Second, reassessment of anatomic location may assist decision making regarding the role of preoperative radiation. Third, information on response to NT may guide the use of adjuvant therapy. For example, Liu *et al*[50] showed that in a cohort of patients in whom CA 19-9 normalized with a decrease > 50% after NT, adjuvant therapy was not associated with additional survival benefit whereas in patients with no normalization of CA 19-9 or decrease of > 50%, receipt of adjuvant therapy was in fact associated with a survival benefit[50]. Additional research is needed to determine whether alternative adjuvant regimens (*e.g.*, gemcitabine-based chemotherapy) should be utilized in patients who do not respond to first-line neoadjuvant chemotherapy (*e.g.*, FOLFIRINOX).

FUTURE DIRECTIONS

Novel biomarkers

With an improved understanding of tumor biology, cancer care is becoming increasingly personalized. Integrated genomic analysis has revealed several molecular tumor subtypes of PDAC as well as subsets of the tumor microenviroment (TME)[57,58]. The clinical applications of such classification systems are still in development but perhaps this data can be used to inform about prognosis or aid in treatment decisions [59]. A shift toward personalized NT will depend greatly on the development and validation of novel biomarkers. As an example, SPARC is a protein that is overly expressed in the TME of PDAC tumors. SPARC expression was associated with an inferior survival in patients receiving gemcitabine-based chemotherapy while no association was detected for patients receiving fluoropyrimidine-based chemotherapy suggesting SPARC expression might act as a negative predictive biomarker in patients treated with gemcitabine-based chemotherapy[60]. However, SPARC expression was not associated with survival or response to gemcitabine-based chemotherapy in patients with metastatic PDAC[61]. Similarly, hENT1 is a receptor that is upregulated on the surface of PDAC cells. Gemcitabine exerts its cytotoxic effects after its cellular uptake by hENT1. Therefore, hENT1 expression could potentially predict therapeutic activity of gemcitabine while its under-expression would be a mechanism for resistance[62,63]. hENT1 was studied as a predictor of response to gemcitabine in the adjuvant setting[64,65], but again could not be validated in the metastatic setting[66]. Additionally, microRNA, serum metabolism profiling, or methylation patterns may prove to be useful biomarkers for diagnosis and potential response to therapy in PDAC[67-69].

Improved methods of measuring response

As systemic therapies for PDAC improves, more novel and sophisticated methods of monitoring tumor response are needed. Response to therapy is routinely based on imaging obtained during the course of treatment. Changes in the tumor-parenchyma interface may serve as an early predictor of response to therapy based on computed tomography imaging[70]. Other novel methods such as detection of circulating tumor cells and circulating tumor DNA (ctDNA) are on the horizon for both diagnosis and markers of response to therapy[71,72]. In patients with PDAC, ctDNA levels have been found to correlate with tumor burden and serial monitoring of ctDNA may provide a method to monitor early response to chemotherapy[73]. Patients who received NT had significantly lower circulating tumor cells compared to patients who were eligible for upfront resection who did not receive NT. Interestingly, alterations in circulating tumor cells were not only observed in response to treatment but also seen before

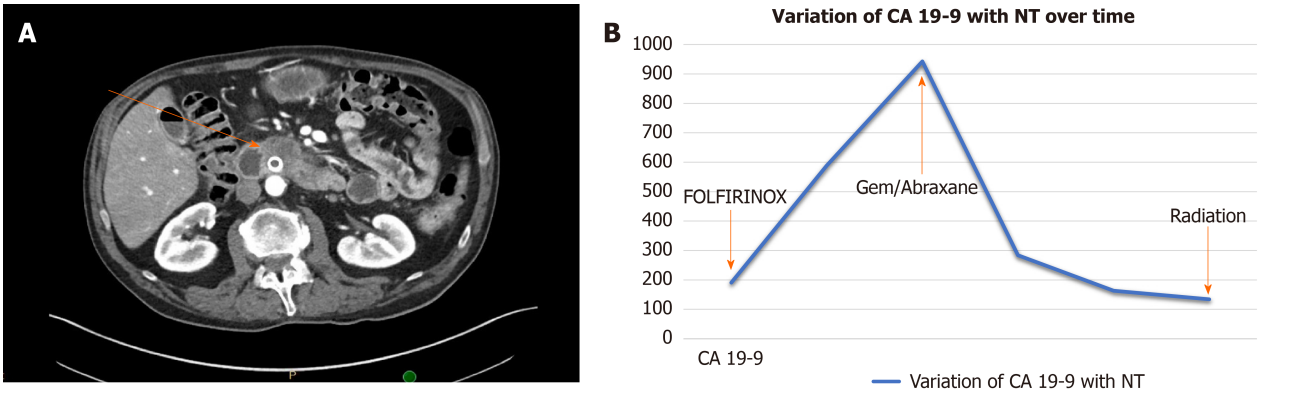


Figure 1 Case example of a 60-year-old man with borderline resectable pancreatic cancer initially treated with FOLFIRINOX. A: Computed tomography image; B: Carbohydrate antigen 19-9 after two months increased so was switched to Gemcitabine/Abraxane with resultant decrease in tumor markers. Given low involvement of superior mesenteric vein, chemoradiation given prior to surgical resection. CA 19-9: Carbohydrate antigen 19-9.

disease recurrence[74]. Future studies will be needed to apply and validate them in the neoadjuvant setting.

Patient-centered outcomes

As the use of NT increases for all stages of PDAC, a greater emphasis on optimizing patient-centered outcomes is necessary and likely to be best achieved through a personalized approach[75]. Despite the advantages of NT, recent evidence has highlighted that some patients will be unable to complete NT and undergo surgery, most commonly because of disease progression or a decrease in their performance status. Recent interest in prehabilitation and advanced nutritional strategies prior to pancreatectomy could be applied to this patient population to improve readiness for surgery[76,77]. Furthermore, although health-related quality of life appears to be preserved during NT, patient symptoms are common during treatment[78]. Prior literature has highlighted the value of monitoring and responding to changes in patient-reported outcomes which could be used to improve and personalize NT[79].

CONCLUSION

PDAC is an aggressive malignancy that is best treated in a multidisciplinary fashion using surgery, chemotherapy, and radiation. NT is an increasingly utilized approach that maximizes the receipt of multimodality therapy, improves margin-negative resection rates, and potentially increases survival durations. In the era of personalized medicine, the neoadjuvant period can also be used to emphasize precision oncology. Already, current methods of anatomically staging, molecularly profiling, and monitoring response to therapy can be used to personalize neoadjuvant treatment for localized PDAC. Ongoing efforts in developing novel biomarkers, innovative methods of measuring response, and patient-reported outcome measurements will expand opportunities for precision oncology during NT. These efforts, along with the development of novel treatment options for this aggressive disease, offer hope for improved multidisciplinary, patient-centered, cancer care.

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MINIREVIEWS

Role of artificial intelligence in multidisciplinary imaging diagnosis of gastrointestinal diseases

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Abstract

The use of artificial intelligence-based tools is regarded as a promising approach to increase clinical efficiency in diagnostic imaging, improve the interpretability of results, and support decision-making for the detection and prevention of diseases. Radiology, endoscopy and pathology images are suitable for deep-learning analysis, potentially changing the way care is delivered in gastroenterology. The aim of this review is to examine the key aspects of different neural network architectures used for the evaluation of gastrointestinal conditions, by discussing how different models behave in critical tasks, such as lesion detection or characterization (*i.e.* the distinction between benign and malignant lesions of the esophagus, the stomach and the colon). To this end, we provide an overview on recent achievements and future prospects in deep learning methods applied to the analysis of radiology, endoscopy and histologic whole-slide images of the gastrointestinal tract.

Key Words: Artificial intelligence; Machine learning; Radiology; Endoscopy; Pathology; Gastroenterology

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Core Tip: Artificial intelligence in general, and machine learning (ML) in particular, have great potential as supporting tools for physicians in the evaluation of neoplastic diseases and other conditions of the gastrointestinal tract. Radiology, endoscopy and pathology images can be read and interpreted using ML approaches in a wide variety of clinical scenarios. These include detection, classification and automatic segmentation of tumor lesions, tumor grading, patient stratification and prediction of treatment response.

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INTRODUCTION

Among all the emerging technologies that are shaping the future of medicine, artificial intelligence (AI) is arguably the one which will most alter the way care is delivered in the short and medium term. There is agreement in the field that AI will deeply impact healthcare, allowing for better diagnostics, better treatments, a more efficient use of the medical resources, and a more personalized management of patients[1,2]. In particular, diagnostic techniques based on medical images are spearheading this revolution[3], because their scope (the analysis of images, which, in general, are already digital), is highly accessible for computing systems. In comparison, other specialties such as emergency medicine or cardiac surgery, are less approachable by computers, and penetration of IA in these medical disciplines will be slower and later.

In the field of gastroenterology, several imaging modalities are used for evaluation of the digestive tract and the diagnosis of gastrointestinal (GI) tumors. These include radiology, endoscopy and histologic sections of GI specimens. In automatic analyses of GI images, a subtype of AI called machine learning (ML) is mainly involved. However, other branches of AI, such as natural language processing (NLP), have also been applied in the field of gastroenterology.

The aim of this review is to synthesize the available evidence on the application of AI to the analysis of radiology, histology and endoscopic images of the GI tract. Other applications of AI in the field of digestive disease, such as NLP, have been reviewed elsewhere[4,5] and are out of the scope of this article.

AI IN A NUTSHELL

AI is an umbrella term referring to different techniques used to solve a given problem by a computing method that mimics human intelligence. This includes automatic image identification, classification and interpretation, as well as recognition and processing of natural language. As such, AI encompasses a wide range of technologies, which vary in their complexity, versatility, and applicability to different types of problems.

Of particular interest is ML, which refers to a specific form of AI that endows computers with the ability to learn from and improve with experience, by-passing the need of being explicitly programmed to perform a specific task[6]. This learning process can be supervised or unsupervised, depending on the existence of labels in the sample dataset used to train the ML algorithms. Supervised learning consists of training an algorithm with classified or labeled examples, while unsupervised learning involves training with unstructured, unlabeled bulk data, and requires that the algorithm extracts the inherent structure thereof[7]. Typical techniques used in supervised learning approaches include support vector machine (SVM), naïve Bayes and random forest (RF). For its part, popular clustering algorithms used for unsupervised learning include k-means, k-medoids and Gaussian mixtures.

Both learning schemes can be used to train artificial neural networks (ANN), a subtype of ML algorithms which have a structure loosely inspired by the human brain.

They are made up of several node units, known as neurons, which are mostly structured in successive layers. The products of one layer go through weighted connections to the entries of the next layer, until a final output is produced. As an example, Figure 1 shows a three-layered neural network: When ANN are composed of many layers of nodes between the input and output, they are called deep neural networks (DNN), and using these models is called deep learning (DL). Thanks to their complexity, DNNs are able to model more complex relationships and execute more difficult tasks.

Convolutional neural networks (CNN) are DNN specialized in image recognition and classification. CNNs automatically extract descriptors by using a special neural network whose weights are determined by a training set. Figure 2 shows the typical structure of a CNN. The scheme shows how the image is consumed by the feature extraction network, which is a neural network composed of subsequent pairs of convolutional and pooling layers. Convolutional layers filter and analyze different image features, which are convoluted with the input data. Then, a pooling layer is used to reduce the size of the image produced by the convolutional layer. This process is repeated as *per* the number of layer pairs in the extraction network. Finally, the classification is done by an ANN acting as a classifier network.

The performance of CNN in the analysis of natural images has already attained similar, and even superior levels compared to that of the human eye[8]. Indeed, thanks to the rise in computational power, the reduction of hardware costs, the substantial development of efficient network architectures and the increasing wealth of data, the last years have witnessed a spectacular surge in AI-driven applications related to medicine.

Today, AI is a hot research topic in many medical fields. As a token, the number of publications found in PubMed using the search term "AI" more than doubled in only 3 years, rising from 6761 in 2016 to 15435 in 2019. The number of results *per* year after adding the term "gastroenterology" to that search also speaks for itself, increasing from 30 to 169 over the same time span (Figure 3).

This illustrates a growing interest among both academic and industry groups in the design and clinical validation of novel applications of AI. This task is facilitated by technology companies, which offer cloud-based services to remotely train DL models. It is expected that ML solutions will become increasingly available through their implementation in computer-aided detection (or diagnosis) (CAD) systems[9], thereby facilitating the widespread usage of these tools in clinical practice. These solutions are becoming progressively offered by the main medical technology vendors, as well as independent clinical software firms.

APPLICATIONS OF AI IN GI RADIOLOGY

Reports of the use of AI in GI radiology are abundant in the literature. Advances in ML have been applied to a wide range of clinical problems related with interpretation of images for diagnosis and decision-making in every anatomic site of the GI tract, from the esophagus to the rectum.

ML models have been trained to process quantitatively mineable features from a wide variety of radiologic modalities, such as computed tomography (CT), magnetic resonance imaging (MRI) and ultrasound (US). Within MRI, virtually any type of sequences, including structural such as T1-weighted and T2-weighted, and functional such as diffusion-weighted imaging (DWI) and apparent diffusion coefficient (ADC) maps, have been incorporated into AI-driven models. In addition, some of the most complex networks have been designed to interpret contrast-enhanced multiphasic CT and MRI images (*i.e.*, dynamic contrast-enhanced MRI series) and even analyze, in a conjoined fashion, multimodal (CT, MR) studies.

Many AI applications focus on, or incorporate steps to automatically segment radiologic images, a task which is very time-demanding when done manually. An array of network architectures is available to provide segmented images of the same resolution of the original ones, but with pixel or voxel-based boundary delimitation of adjacent tissue types[10].

The use of ML and DL algorithms is, in many instances, accompanied by radiomic approaches. Radiomics, a term which was coined as late as 2012[11], refers to the quantitative and objective analysis of features in radiologic images by computational means, in order to gain insight into otherwise invisible, or hardly quantifiable information of potential clinical relevance. A cornerstone of radiomic assessments involves the analysis of image texture features. Radiologic textures (Figure 4) refer to

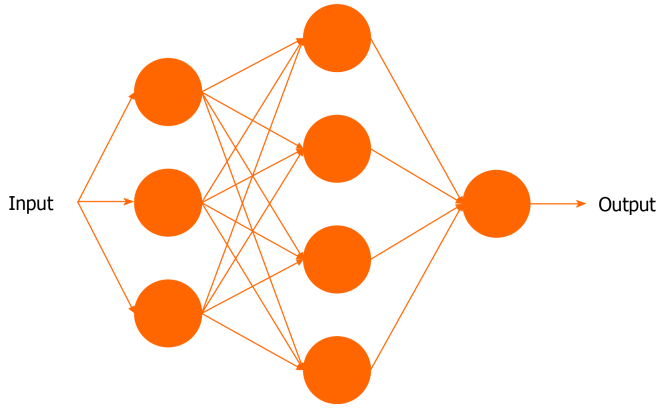


Figure 1 A neural network made up of 3 layers: An input layer, a hidden layer and an output layer. The input data is taken by the neurons (shown as blue circles) in the input layer, and produce an output which is consumed by the next layer (hidden layer). They, in turn, perform similar computations and provide an output to the output layer, which yields a final output.

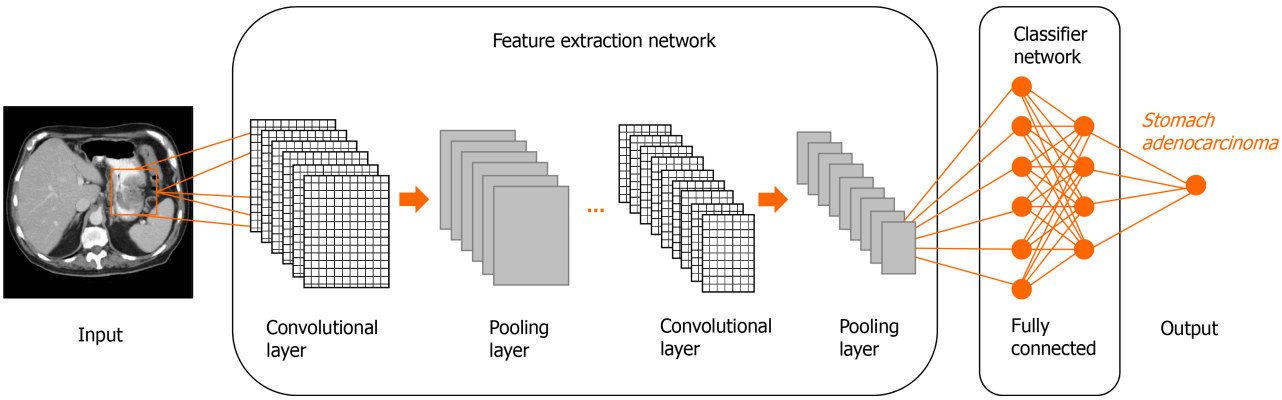


Figure 2 Conceptual example of a simple convolutional neural network used for classification of a stomach tumor.

differences in the grayscale intensities of adjacent pixels or voxels within a region of interest, and have been associated with intratumoral heterogeneity[12]. Radiomics is closely linked to IA, because it often uses ML methods to discover patterns in large datasets[13], although, alternatively, statistical approaches can be used instead.

In the following sections, we review the currently reported experience in the AI-based analysis of GI radiology studies in different clinical scenarios.

Esophageal neoplasia

Esophageal cancer (EC) is a very aggressive neoplasm, with two main histological subtypes: Squamous cell carcinoma (ESCC) and adenocarcinoma. Currently, EC is preferably treated with chemoradiation (CRT), with a positive correlation between the histopathological response and overall survival[14]. However, common derived parameters from CT and positron emission tomography (PET) have shown a limited accuracy in treatment prediction and response assessment. In this scenario, radiomics and DL methods have shown initial promise in prediction of response to treatment in patients with EC. Radiomic signatures in PET images have been used to predict prognosis in patients with esophageal adenocarcinoma[15] and in patients with ESCC [16]. Also, ^{18}F-fluorodeoxyglucose (FDG) PET/CT metrics and textural features showed utility in predicting response to induction chemotherapy followed by neoadjuvant CRT in a mixed cohort including patients with either type of EC[17].

Prognosis prediction of patients with EC has also been addressed from the viewpoint of CT. For instance, Ou *et al*[18] used multivariable logistic regression, RF and SVM classifiers on radiomic biomarkers extracted from CT data to predict resectability of ESCC[18]. Also, Jin *et al*[19] evaluated the potential of an integrated model combining radiomic analyses on CT images and dosimetric parameters in predicting response to CRT in patients with esophageal adenocarcinoma or ESCC. The combined model achieved an accuracy of over 0.7, and displayed a better prediction performance than the model using radiomic features alone[19]. Later, Hu *et al*[20] investigated the

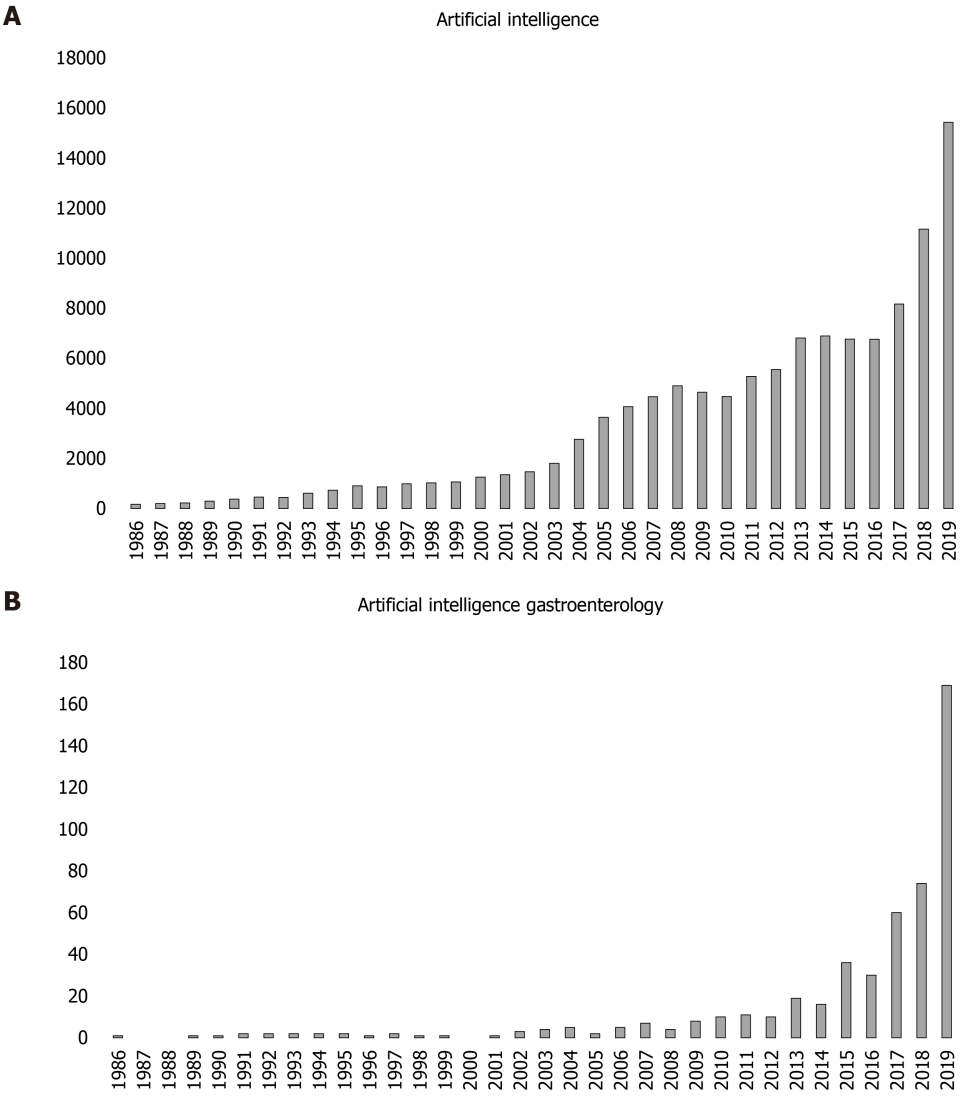


Figure 3 PubMed results by year using the search terms. A: Artificial intelligence; B: Artificial intelligence gastroenterology.

role of radiomic features and DL models in predicting response to neoadjuvant CRT in patients with ESCC eligible for surgery. Manual radiomic feature extraction and feature mapping by CNN were done on pre-treatment CT images. DL modeling based on SVM classifiers showed a greater capacity in assessing tumor heterogeneity and outperformed handcrafted radiomic markers in predicting pathologic complete responses of the tested cohort. Of note, the combination of radiomics and DL did not result in a better predictive performance than DL alone[20]. CT radiomics features have also been successfully used to identify programmed death-ligand 1 and CD8+ tumor infiltrating lymphocyte expression levels in patients with ESCC. Such analyses, performed on pretreatment CT images, allowed for better patient stratification and selection of candidates for immune checkpoint inhibitor therapy[21]. Finally, other encouraging applications of CT radiomics analysis in ESCC are pretreatment local staging[22] and assessment of lymph node status[23,24] with advantage over morphological features and size criteria.

In addition, radiomics analysis has also been applied to MRI, although this technique is challenging to perform in the esophagus due to motion artifacts. Preliminary results of radiomics analyses using pretreatment T2-weighted sequences show promise in the prediction of treatment response to CRT in patients with ESCC [25].

Gastric adenocarcinomas and GI stromal tumors

Radiomic analyses with different techniques have been applied to improve malignant tumor detection and characterization in the stomach. In this regard, a nomogram including multiphasic CT radiomics features was able to predict the presence of gastric adenocarcinomas[26].

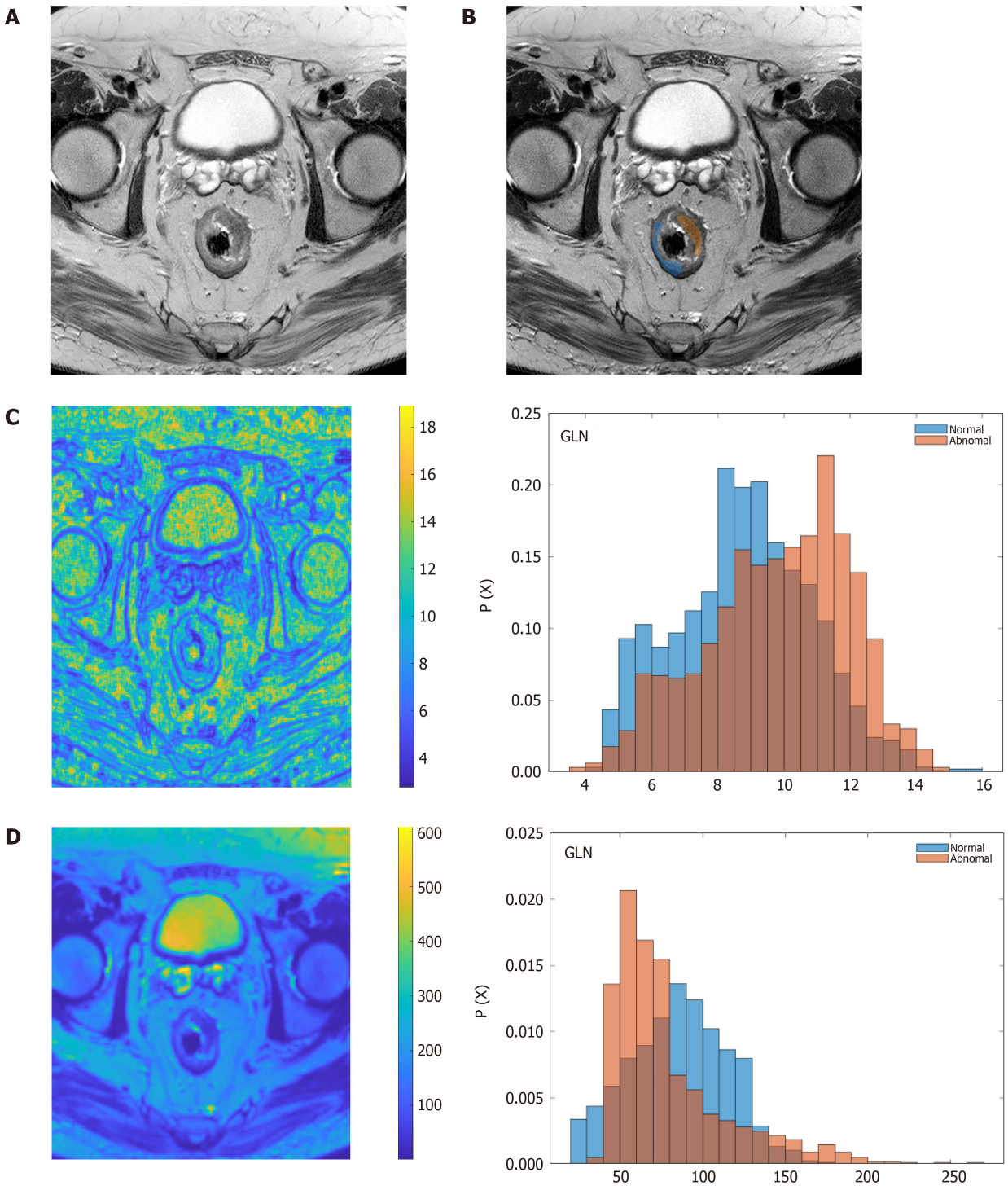


Figure 4 Texture analysis of rectal adenocarcinoma. A: Original, axial T2-weighted image; B: Region of interest delineation of rectal tumor mass (orange) and normal tissue (blue); C and D: Parametric images and histograms of two different texture descriptors, showing differences between normal (blue) and tumor (brown) regions; C: Grey-level nonuniformity; D: High gray-level run emphasis. GLN: Grey-level nonuniformity; HGRE: High gray-level run emphasis.

The aggressiveness potential of gastric adenocarcinomas can be predicted by ML and radiomic analyses on CT and MRI of the stomach. Texture analysis performed in portal venous phase CT scans discovered feature correlates of lymphovascular and perineural invasion potential in patients with tubular gastric adenocarcinomas treated with total gastrectomy. Classifications done with eight different ML models were evaluated, among which naïve Bayes and RF exhibited the best performance in predicting the existence of lymph nodes metastasis, vascular and perineural invasion [27]. Similarly, quantitative assessment of intratumoral heterogeneity of gastric adenocarcinomas by entropy parameters extracted from ADC maps could be correlated with overall stage and prognostic factors of malignant behavior, including vascular and perineural invasion[28]. Another very recent study has shown the

potential role of a double-energy CT radiomics nomogram for prediction of lymph node metastases in gastric adenocarcinomas, with advantages over the current clinical model. In addition, the nomogram demonstrated a significant correlation with patient survival[29]. Also, AI and radiomics analyses have been applied to the prediction of treatment response of metastatic gastric adenocarcinomas. An ANN predictive model using radiomics analyses of pretreatment contrast-enhanced computed tomography (CECT) demonstrated significant differences between responders and non-responders, in a cohort of patients treated with pulsed low dose radiotherapy[30].

In addition, ML has been successfully applied to diagnosis and clinical decision-making of therapeutic strategies in patients with GI stromal tumors (GIST). As reported by Wang *et al*[31], ML showed the ability to differentiate between gastric schwannomas and GISTs by assessing CT images[31]. In that study, radiologists performed worse than all of the five different ML models tested, which included RF, logistic regression and decision trees. Also, analysis of CECT scans by a residual neural network has proved useful to predict the risk of recurrence after curative resection of localized primary GIST[32]. This offers new avenues for non-invasively distinguishing between patients with high risk of recurrence, for which adjuvant treatment with imatinib is recommended, and low-risk patients eligible for curative resection who are not likely to benefit from imatinib adjuvant therapy.

Colorectal cancer

AI has been extensively researched in the detection, characterization and staging of colorectal cancer (CRC)[33]. Several ML techniques have shown utility in the automated detection of polyps by CT colonography[34,35]. Also, radiomic analyses of colorectal CT images were successful in classifying CRC lesions according to their *KRAS* gene mutation status[36].

Currently, MRI is considered the most accurate test for rectal cancer staging[37]. A faster region-based CNN was trained to detect metastatic lymph nodes in T2-weighted and DWI images of the pelvis. The N staging provided by this network was very consistent with that done by radiologists, while average diagnostic time was markedly shorter (20 ms *vs* 600 ms, respectively)[38,39].

In addition, radiomic models aided by SVM were useful in predicting liver metastases of colon cancer. A combined model which included radiomic features of preoperative CT scans with two clinical variables (tumor site and diameter of tumor tissue) showed a higher prediction performance than either the clinical features or the radiomic signatures taken separately[40].

Radiomics analyses of CT, MRI and PET/CT images have been extensively used to predict treatment outcome and survival in patients with CRC. A recent systematic review analyzed 81 studies focused on this task, finding only 13 high-quality reports demonstrating a good performance in predicting treatment response, which mainly involved MRI studies of rectal cancer. Of note, the authors concluded that radiomics research in this field needs more clinical validation, rather than new algorithms[41]. Van Helden *et al*[42] discovered radiomic predictors of response to palliative systemic therapy and survival of patients with metastatic CRC, using pre-treatment ^{18}F-FDG PET/CT images processed with semiautomatic segmentation[42].

Also, in this line, a recent report investigated the prognostic value of a ML model based on liver CT radiomics analyses to predict survival of patients with metastatic CRC[43]. Interestingly, CT-based prediction models were superior to their clinical counterparts for 1-year survival prediction.

Radiomic analyses may be useful to guide treatment of locally advanced rectal cancer (LARC). A radiomic signature comprising 10 texture features from T2-weighted images were modeled by a RF algorithm and used to evaluate treatment responses of subjects with LARC after neoadjuvant chemotherapy. The signature showed good prediction of patient response to treatment, with a good discrimination power among complete responders, partial responders and non-responders, with areas under the curve (AUC) in the 0.83-0.86 range[44].

In another study, Cui *et al*[45] developed and validated a radiomic signature based on 12 features extracted from multiparametric MR images [T2-weighted, dynamic contrast enhanced (DCE) and ADC maps] to predict complete response in patients with LARC after neoadjuvant CRT. The combination of all three image types showed a better predictive value than either of them alone, with an AUC of over 0.94[45]. This was hypothesized to result from the different aspects of tumor behavior reflected by each modality, such as tumor intensity, vascularization and cellularity, respectively.

Segmentation of radiologic images

Segmentation is a key step in the diagnosis and evaluation of tumor diseases, as well as in the treatment volume calculation for radiotherapy planning. However, manually delineating the relation between tumors and adjacent structures is a very time-consuming task for radiologists, requires a considerable level of expertise, and often lacks adequate inter-observer reproducibility.

Several DL models have been developed for automatic delimitation of tissue boundaries, many of which have been specifically tested in GI sites. Of note, automatic segmentation can be particularly challenging in the abdomen, due to the lack of clear boundaries between some organs which are displayed with similar intensities in this anatomy, such as the liver, the stomach, the spleen and the kidneys. Also, peristaltic and breathing motions make the assessment of the GI tract with US and MRI more difficult.

The complexity of networks involved in image segmentation varies. Today, the more recent and sophisticated networks are based on fully convolutional networks (FCN), which are optimal frameworks for semantic image segmentation. Popular FCN-based architectures include SegNet[46] and U-net, a CNN specialized in fast and accurate segmentation of biomedical images[47]. U-net networks can be trained with a relatively limited set of data and are a usual choice for many automatic segmentation approaches.

Automatic segmentation of rectal tumors has been attained using T2-weighted[48,49], DCE[50] and multiparametric (T2, DWI) MRI[51]. Multi-organ, and even full-abdomen segmentation are feasible, although they usually rely on multi-atlas label fusion[52]. This strategy requires registration and fusion of images acquired at different levels of the same patient and is prone to error due to inefficient image registration. Nonetheless, Gibson *et al*[53] recently proposed a registration-free method for multi-organ segmentation of CT images, based on a FCN called DenseVNet[53].

A useful application of automatic segmentation is the calculation of the clinical target volume of tumors in planning CT images for radiotherapy purposes. This approach has the advantage of yielding more reproducible measurements of treatment areas, leading to more consistent radiation doses, and has been explored in the assessment of EC[54] as well as rectal tumor lesions[55,56], among others.

APPLICATIONS OF AI IN ENDOSCOPIC TECHNIQUES

AI in GI endoscopy holds tremendous promise to augment clinical performance, establish better treatment plans, and improve patient outcomes.

In September 2019, the first multidisciplinary global gastroenterology AI meeting (First Global AI in Gastroenterology and Endoscopy Summit) was held in Washington, DC (United States), with a mandate to discuss and deliberate on practice, policy, ethics, data security, and patient care issues related to identification and implementation of appropriate use cases in gastroenterology[57]. Among the many challenges related to the use of AI for gastroenterology applications, Summit attendees highlighted 7 future needs to advance the field of AI in this discipline (Table 1).

Today, a wide variety of AI applications in GI endoscopy are being proposed, developed, and, in some cases, validated in clinical trials. The most current AI algorithms for gastroenterology are in the field of computer vision, which refers to technologies that can "see and interpret" visual data, such as a live video stream from the endoscope.

Polyp detection

Computer-aided polyp detection (CADe) and computer-aided polyp diagnosis (CADx) are two applications of AI-assisted computer vision for colonoscopy, which are already extensively studied[58,59]. CADe and CADx have been the areas of most rapid progress so far in applying AI/computer vision to GI endoscopy. Given the central importance of screening and surveillance colonoscopy for CRC prevention, continued development and validation of CADe and CADx remains a top priority.

AI-assisted polyp detection studies must apply validated outcome parameters, such as adenoma detection rate, adenomas *per* colonoscopy, or adenoma miss rate, among others. Most of these studies are early-stage, but show promising clinical relevance. For instance, a recent study showed that an AI system based on DL and its real-time performance led to significant increases in polyp detection rate[60].

Table 1 Future needs to advance the field of artificial intelligence in gastroenterology

No.	Future needs
1	Identification of relevant and well-defined use cases
2	Development of high-quality metrics
3	Creation of large-scale imaging datasets
4	Clarity on the regulatory path to market
5	Use of appropriate AI methods
6	Clarification of patient privacy issues
7	Education of gastroenterologists on the risks and benefits of AI

AI: Artificial intelligence.

Computer vision and image classification

Prioritization of additional use cases for computer vision in GI endoscopy must consider the prevalence of the targeted disease state, the possible clinical impact of the proposed algorithm, and the potential solvability of the clinical problem by AI.

The most common GI cancers share a common natural history. In this manner, CRC, gastric cancers, and ECs have precursor lesions that can be diagnosed by traditional endoscopic modalities. Additionally, inflammatory bowel disease (IBD) represents a relatively high prevalence group of conditions with an elevated risk of CRC but with dysplastic precursor lesions that are difficult to recognize endoscopically.

A major barrier to progress in ML in GI endoscopy is the relative absence of high-quality, labeled images for training AI algorithms. To overcome this, there is an urgent need to develop rules and recommendations regarding appropriate formats and quality standards for endoscopy image and video, along with accepted protocols for categorizing such images, storing metadata, and transferring and storing images while protecting private health information.

Image classification using AI has been developed to detect gastric cancer in endoscopic images with an overall sensitivity of 92.2%[61]. Another study applied a CNN to quantify the invasion depth of gastric cancer based on endoscopy data[62]. Similarly, a CNN was constructed to characterize EC, achieving a sensitivity as high as 98%[63].

GI bleeding

DL is especially relevant to video capsule endoscopy, given the large amount of data (around 8 h of video) and low efficiency of manual review by the physician. AI tools have also been shown to detect small-bowel bleeding and other relevant images most likely to be clinically relevant. The first studies involving CAD of bleeding from video capsule endoscopy images used color and texture feature extraction to help distinguish areas of bleeding from area of nonbleeding[64]. More recent studies, including those published by Jia and Meng[65], and Hassan *et al*[66], used DL-based features to achieve sensitivities and specificities as high as 99% for the detection of GI bleeding.

Shung *et al*[67] recently published a systematic review summarizing the use of ML techniques to predict outcomes in patients with acute GI bleeding. Fourteen studies with 30 assessments of ML models were included in their analysis. They predicted that ML performed better than clinical risk scores for mortality in upper GI bleeding (UGIB). Overall, the AUC for ANNs (median, 0.93; range, 0.78-0.98) was higher than other ML models (median, 0.81; range, 0.40-0.92)[67].

The emergence of automated lesion recognition endoscopy software combined with the boost in robotic techniques in surgery[68] has captured the attention of GI endoscopists and surgeons. For example, clinical risk-scoring systems offer invaluable, but not always practical, help for better stratification of patients at risk of UGIB and hemodynamic instability. Seo *et al*[69] developed an ML algorithm that predicts adverse events in patients with initially stable non-variceal UGIB[69]. Primary outcomes analyzed in this study included adverse events such as mortality, low blood pressure and rebleeding within 7 d. The authors compared four ML algorithms (logistic regression with regularization, RF classifier, gradient boosting classifier and voting classifier) with clinical Glasgow–Blatchford and Rockall scores. They found that the RF model achieved the highest accuracy and offered significant improvement over

conventional methods used for mortality prediction.

IBD and AI

Recently, AI solutions have been explored to better define mucosal healing in IBD[70]. With the incidence and global burden of IBD still on the rise, involving a large number of young patients with normal life expectancy, there is a constant need for more accurate ways to stratify risks and predict prognosis in these patients. Because the concept of mucosal healing is a rather new concept, the authors expect that AI tools for healing evaluation through endoscopic monitoring, which have recently been developed, could play a key role in better standardizing it[70].

Waljee *et al*[71] used RF methods to develop and validate prediction models of remission in patients with moderate to severe Crohn's disease. In 401 participants, they showed an AUC of 0.78 at week 8 and an AUC of 0.76 at week 6. Also, Klang *et al*[72] reported on the training of a CNN to detect Crohn's disease ulcers, using 17,640 capsule endoscopy images from 49 patients[72].

Assessment of ulcerative colitis (UC) can sometimes be very difficult. In early stages of the disease, erythema can be attributed to a number of other conditions, delaying UC diagnosis. On the other hand, foci of dysplasia in advanced disease are often missed due to the small amount of colonic mucosa sampled in surveillance colonoscopy. Thus, the development of AI solutions aimed at helping assess UC activity is a hot research topic.

For instance, Gutierrez *et al*[73] proposed an automated end-to-end system using DL to binary predict the Mayo Clinic Endoscopic subscore, which showed a high degree of precision and robustness, with an AUC of 0.84[73]. In this connection, Kirchberger-Tolstik *et al*[74] recently reported a non-destructive biospectroscopy technique assisted by neural networks to assess the severity of the disease according to the Endoscopic Mayo Score, with a mean sensitivity of 78% and a mean specificity of 93%[74].

Maeda *et al*[75] developed and evaluated a CAD system for predicting persistent histologic inflammation using endocytoscopy. To do so, they classified the endoscopic studies according to the histological findings of the corresponding biopsies, obtaining a predictive model with a high specificity and sensitivity[75]. In addition, Bossuyt *et al*[76] constructed an algorithm tracking mucosal redness density in the red channel of endoscopic images along with vascular patterns. The results were accurately correlated with the activity of the disease at the endoscopic and histological level[76].

APPLICATIONS OF AI IN GI PATHOLOGY

Today, most pathology labs still rely on fully analogic workflows, including the use of optical microscopes. However, with the availability of digital slide scanners clearly on the rise, there is a growing interest in the development of AI applications for histopathological studies.

Computational analyses of whole-slide images (WSI) of histology sections are regarded as a very promising form of improving diagnostic accuracy, reducing turnaround times and increasing interobserver agreement. Some studies regarding ML applications in pathology are discussed in this section.

Esophageal neoplasia

AI can be used for detection and grading of tumor lesions in the esophagus, and in particular of Barrett´s esophagus (Figure 5). Early detection of this condition is essential to improve patient prognosis. Histopathology is currently the gold standard for the diagnosis of this entity, but its study is characterized by a low degree of interobserver agreement in dysplasia grading.

To overcome this problem, Sabo *et al*[77] developed two computerized morphometry models based on size, shape, texture, symmetry and distribution analyses of the epithelial nuclei of the esophagus. The first neural network model showed an accuracy of 89% in the differentiation of normal esophagus *vs* esophagus with low-grade dysplasia. The second model distinguished between low-grade and high-grade dysplasia with an accuracy of 86%, demonstrating potential for assisting pathologists in the differential diagnosis of indistinguishable lesions[77]. Van Sandick *et al*[78] and Polkowski *et al*[79] also addressed this issue, by combining morphometric analyses in hematoxylin and eosin (H&E) stains with immunohistochemistry data for p53 and Ki67. This hybrid approach improved the accuracy of the differentiation between low-grade and high-grade dysplasia up to 94%. Lastly, Sali *et al*[80] compared the performance of three different models in classifying precursor lesions of Barrett's

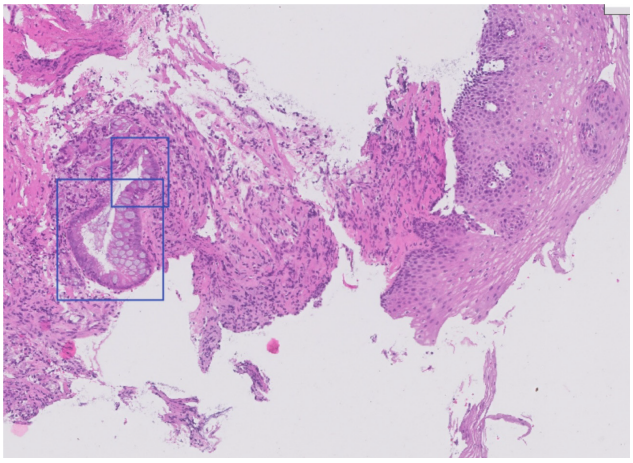


Figure 5 Automatic detection of intestinal metaplasia in a sample of esophagus tissue stained with hematoxylin and eosin. Image analyzed with research software from Cells IA (https://cells-ia.com/).

esophagus, which differed in their training approach (supervised, weakly supervised and unsupervised). The CNN trained with an unsupervised approach extracted the most relevant image features for identifying and classifying the cancer precursors[80].

Inflammatory and infectious lesions of the stomach

Chronic gastritis is a very prevalent condition. Its diagnosis is established by evaluating the degree of chronic and active inflammation, presence of *Helicobacter pylori* (*H. pylori*), atrophy of the mucosa and intestinal metaplasia. Steinbuss *et al*[81] used the Xception CNN architecture for automatic classification of three types of gastritis, namely type A (autoimmune), type B (bacterial), and type C (chemical) gastritis, in histological sections of antrum and corpus biopsies, with an overall accuracy of 84%[81].

H. pylori cells can be visualized in histology sections of gastric biopsy samples using different staining techniques, such as H&E, Giemsa or Warthin-Starry silver stains (Figure 6). Klein *et al*[82] published a DL algorithm for automatic *H. pylori* screening in Giemsa stains, with a sensitivity of 100% and a specificity of 66%[82]. In parallel, Zhou *et al*[83] used a CNN to assist pathologists in the detection of *H. pylori* cells in H&E-stained WSI, but failed to demonstrate significant improvements in diagnostic accuracy and turnaround times in comparison with unassisted case studies[83].

Gastric cancer

Early detection and histopathologic characterization of gastric tumors are essential to improve treatment outcomes. A number of recent studies have paid attention to lesion detection, classification and characterization in this anatomic site. For instance, Song *et al*[84] developed and trained a deep CNN to differentiate between benign and malignant gastric tumors, with a sensitivity of 100% and a specificity of 80.6%[84]. Also, a network developed by Sharma *et al*[85] classified gastric cancer cases according to immunohistochemical response and presence of necrosis, with accuracy rates of 0.699 and 0.814, respectively[85].

Mori and Miwa[86] used a 6-layer CNN to study tumoral invasion depth in gastric adenocarcinoma images, with an accuracy of 85%, a sensitivity of 90% and a specificity of 81%[86]. Later, Iizuka *et al*[87] trained CNN and recurrent neural networks to classify stomach and colon WSI into adenocarcinoma, adenoma and non-neoplastic, with AUC values up to 0.97 and 0.99 for adenocarcinoma and gastric adenoma, respectively[87].

Lastly, Kather *et al*[88] trained a DL model to predict microsatellite instability from H&E-stained gastric cancer images, using a pathomics approach. This represents a very innovative approach, which extracts molecular and histochemical features solely based on H&E images and may circumvent the need to conduct genetic and/or immunohistochemical tests[88].

Colonic inflammatory disease, polyps and CRC

Klein *et al*[89] evaluated the histomorphometric features of colon biopsies from patients with Crohn's disease. These analyses revealed that differences in the number

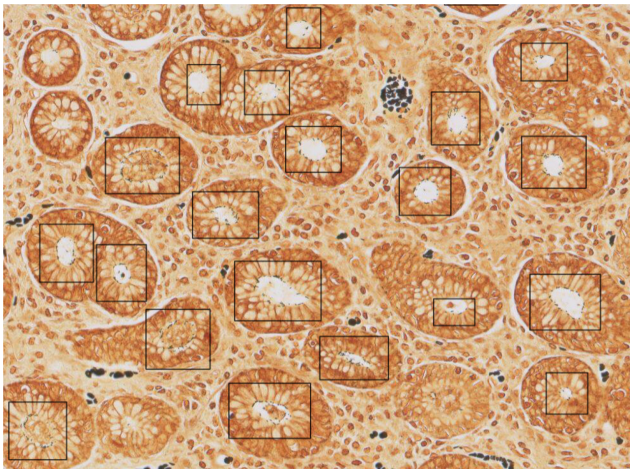


Figure 6 Automatic detection of *Helicobacter pylori* infection in a gastric biopsy section stained with Warthin-Starry stain. Image analyzed with research software from Cells IA (https://cells-ia.com/).

of inflammatory cells, lymphocytic aggregates and collagen density can be used as predictors of clinical phenotypes with an accuracy of 94%[89].

CRC is among the most common malignancies and a major cause of cancer-related death worldwide. Today, it is known that the vast majority of CRC cases arise from the adenoma-carcinoma sequence[90], and early detection of these lesions is considered of utmost importance to reduce CRC incidence rates. Despite this, evidence concerning the application of DL techniques to detect and characterize precancerous lesions in WSI of this anatomic site is still scarce.

Rodriguez-Diaz *et al*[91] developed a DL model to locate areas of malignant transformation inside polyps using semantic segmentation, distinguishing between neoplastic and non-neoplastic polyps, with a sensitivity of 0.96 and a specificity of 0.84 [91]. Haj-Hassan *et al*[92] used a CNN to classify segmented regions of interest into three tissue types related to CRC progression (benign hyperplasia, intraepithelial neoplasia and carcinoma), with an accuracy of 99.17%[92]. Korbar *et al*[93] applied a residual network architecture to classify five polyp types (hyperplastic, sessile serrated, traditional serrated, tubular, and tubulovillous/villous) on WSI with an overall prediction accuracy of over 93%[93]. Also, several studies have proposed DL predictive models of survival for patients with CRC based on the extraction of prognostic markers in H&E images[94,95].

CONCLUSION

In this review, we have summarized the current evidence on the application of AI to the interpretation of radiology, endoscopy and histological images of the GI tract. At this moment, data on the use of AI in the assessment of radiology images of the GI are far more abundant than those from endoscopy and pathology studies. This difference is mainly a consequence of the asymmetrical availability of data and degree of digital transformation of each specialty. In any case, taken together, the available body of knowledge allows us to anticipate the central role that can be expected for AI in the personalized management of patients with high-risk GI tumors and other GI conditions.

On the downside, a large proportion of papers published to date are proof of concept approaches, based on retrospective analyses and single-center studies, usually involving limited data. As a result, and although there is growing evidence on its applicability in many clinical scenarios, the actual contribution of AI to clinical care in gastroenterology is still very limited.

The next several years will likely be a period of rapid development for AI tools in gastroenterology imaging. It is crucial that, as this field advances, there is a focus on technologies that provide real clinical benefits that have been validated in high-quality clinical trials. To this end, prospective, multicenter studies involving large sets of real-world data that reflect the high variability of image quality among institutions, will be key to assess the actual clinical applicability of AI solutions in this field.

As the development and uptake of AI in gastroenterology continues to grow, we will likely see a shift in the way diagnoses of GI conditions rely on AI. Evidence on the superior performance of computers over human experts in most diagnostic scenarios involving image interpretation will soon be notorious. This might ultimately lead to a deskilling of specialists, who would potentially suffer from too great a reliance on AI for their diagnoses, a situation which might be aggravated by the "black-box" nature of many AI applications. Indeed, radiomics and pathomics approaches, which use imaging features that are otherwise invisible to the eye, or highly subjective, have shown a clear value in identifying patient profiles with higher risk, even before clinical symptoms appear. This can facilitate personalized treatment and improve the prognosis of patients with GI conditions, but efforts should be made to increase the "explainability" of AI-rendered results to both physicians and patients, in order to facilitate broad readership and understandability of diagnoses.

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MINIREVIEWS

Hyperbaric oxygen therapy as a complementary treatment for radiation proctitis: Useless or useful? – A literature review

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Abstract

Radiotherapy (RT) is the backbone of multimodality treatment of more than half of cancer cases. Despite new modern RT techniques, late complications may occur such as radiation proctitis (RP). The natural history of RP is unpredictable. Minor symptoms may resolve spontaneously or require conservative treatment. On the other hand, for similar and uncomplicated clinical contexts, symptoms may

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persist and can even be refractory to the progressive increase in treatment measures. Over the last decades, an enormous therapeutic armamentarium has been considered in RP, including hyperbaric oxygen therapy (HBOT). Currently, the evidence regarding the impact of HBOT on RP and its benefits is conflicting. Additional prospective and randomised studies are necessary to validate HBOT's effectiveness in the 'real world' clinical practice. This article reviewed the relevant literature on pathophysiology, clinical presentation, different classifications and discuss RP management including a proposal for a therapeutic algorithm with a focus on HBOT.

Key Words: Radiation proctitis; Radiation proctopathy; Radiotherapy; Radio-induced lesion; Late radiation tissue injury; Delayed radiation injury; Late sequelae; Hyperbaric oxygen therapy; Hyperbaric oxygen; Review

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Core Tip: Over the last decades, an enormous therapeutic armamentarium has been considered in radiation proctitis (RP) management including hyperbaric oxygen therapy (HBOT). However, evidence regarding the impact of HBOT on RP and its benefits is conflicting. With the lack of consensus to guide the use of HBOT for the treatment of RP, the goal of this review was to synthesise the existing data, analyse results of previous studies, identify gaps in knowledge, and discuss RP' management including a proposal of a therapeutic algorithm focusing on HBOT.

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INTRODUCTION

In recent years and on a global scale, we have witnessed a sustained increase of cancer incidence, with a steady growth rate of approximately 3% per year. According to the International Agency for Research on Cancer of the World Health Organization, it is estimated that worldwide the number of new cancers will reach almost 30 million in 2040, with a mortality of 16.5 million[1].

However, these alarming numbers may be related to the implementation of screening programs with the detection of more cases of cancer at an early stage. Moreover, the survival rates of patients diagnosed with cancer have also increased, largely due to scientific development and the commitment of health professionals in oncology. The progressive increase of this new population cancer survivors with specific clinical and social problems poses a real healthcare challenge. It is in this particular context that radio-induced lesions arise, taking into account their prevention and treatment.

In developed countries, radiotherapy (RT) is the backbone of multimodality treatment of more than half of cancer cases. Despite new modern RT techniques, delayed radiation injury can appear with a latency period of a few months to several decades. The incidence and prevalence of radio-induced lesions and their severity are not well known due to different definitions, underestimation of mild symptoms by both patients and professionals, and the imprecise notification of their appearance in clinical practice[2].

RT is a key treatment of the multimodal approach of neoplasms of the gastrointestinal and pelvic regions. The fixed anatomical position of the rectum in the pelvic brim and the proximity to the irradiated organs makes the rectum especially vulnerable to secondary ionising radiation injury[3]. Radiation proctitis (RP) or radiation proctopathy is defined as a chronic lesion of the mucosa and submucosa of the rectum or rectosigmoid transition secondary to ionising radiation[3].

Acute radio-induced lesions occur from hours or days after exposure to ionising radiation and usually resolve in less than 3 mo. On the other hand, RP is a late lesion with a median onset of 6 to 12 mo after exposure. Rare cases with a latency over 30 years have been reported. Furthermore, RT can develop a continuous process from an acute injury, where symptoms are not specific or non-existent. The clinical presumption is based on intestinal symptoms (*e.g.*, haematochezia, diarrhoea, tenesmus, abdominal pain) and in a cause-effect relationship of previous history of pelvic RT. The diagnosis can be confirmed by endoscopy with or without histologic examination, as imaging findings are usually nonspecific[4-12].

Virtually all patients will experience some clinical manifestation of acute RP during their pelvic RT treatment[8]. Previously, it was thought that only a minority of patients (5%-15%) would develop RP[9,13,14]. However, based on recent data, it is now estimated that near half of the patients may report symptoms related to RP[14,15]. Following RT, 30% of patients with prostate cancer, 12%-17% with rectal cancer, 16% with testicular cancer and 10% with cervical cancer will develop RP[16]. RP's most severe cases have an estimated 4.3%-22% incidence and a 2%-8% mortality rate[11-16].

A high risk of developing RP depends on the total radiation dose and its fractionation, the mode of application (external *vs* intracavitary), the volume of irradiated tissue and the combination of RT techniques. A cumulative dose of RT < 45 Gy is associated with a lower risk of late RT lesion in contrast to what is observed for doses > 70 Gy, for which the risk is significantly higher. The RT technique used is an essential predictor of risk for RP. Compared to brachytherapy, ionising radiation typically administered through a linear accelerator will result in greater and more significant exposure of the contiguous organs. The new RT modalities that comprise three-dimensional conformational RT, intensity-modulated RT and proton and neutron therapies seem to be associated with a lower risk of gastrointestinal toxicity[5-9,17,18].

Advanced age, low body mass index, smoking habits, previous abdominal surgery due to intraperitoneal adhesions, pelvic inflammatory disease, arterial hypertension, diabetes mellitus, previous chemotherapy, collagen and vascular diseases, xeroderma pigmentosa, Cockayne syndrome are other patient-related factors that may be associated with a higher RP risk. Among this array of risk factors, those that seem to have the most significant predictive value for RP are the history of abdominal surgery, chemotherapy, arterial hypertension, and thinness[5-13].

Although the processes of obliterating endarteritis, hypoxia, and fibrosis are already recognised as fundamental factors for their establishment and eventual evolution to chronicity, RP's pathophysiology is complex and has not yet been fully understood[12,19-21]. This chronic condition can stabilise or gradually worsen with periods of acute inflammation.

The beneficial properties of hyperbaric oxygen, together with the growing knowledge about the pathophysiology of delayed radiation injuries, have led to the use of hyperbaric oxygen therapy (HBOT) in the treatment of RP[4]. Currently, HBOT is considered by the European Committee for Hyperbaric Medicine (ECHM) as a treatment modality for late radio-induced lesions, namely, the prevention and treatment of osteoradionecrosis of the mandible, haemorrhagic radiation cystitis and RP (degree of recommendation I/level of evidence B). Although HBOT is used in selected cases of other RT sequelae (*e.g.*, central nervous system and radio-induced laryngeal lesion), its degree and level of recommendation/evidence is lower[22].

Attending to the lack of consensus on guidelines for the use of HBOT in the treatment of RP, the goal of this review is to synthesise the existing data, analyse results of previous studies, identify gaps in knowledge, and discuss RP' management including a proposal for a therapeutic algorithm with a focus on HBOT.

RP PATHOPHYSIOLOGY

Ionising radiation can cause cellular damage, especially in mucosa with rapid renewal such as the intestinal mucosa.

Acute lesions occur predominantly in the mucosa, which consists of depletion of epithelial cells due to cytotoxicity in progenitor cells and consequent apoptosis; inflammation and infiltration of the lamina propria with polymorphonuclear leukocytes and plasma cells; eosinophilic abscesses of intestinal crypts; endothelial lesions of intestinal microvascularisation and eventual oedema of the submucosa. These processes result in mucositis that interferes with the intestinal barrier's function, allowing antigen translocation including microorganisms[23]. If the submucosa

modifications are not impactful, the epithelial cells regenerate and the process resolves spontaneously (Figure 1).

Parallel to what has been described for other late sequelae of RT, RP's pathophysiological mechanisms are not fully understood. Oxidative stress caused by reactive oxygen species (ROS) contributes to obliterating endarteritis with progressive reduction of parietal irrigation and consequent local ischaemia, formation, and diffuse progression of mucosal and submucosal fibrosis through a local proinflammatory cytokines cascade (high levels of interleukin-1β (IL-1β), IL-2, IL-6, IL-8, and transforming growth factor-β), which is promoted by macrophages, neutrophils, and by the differentiation of fibroblasts into myofibroblasts (Figures 1 and 2)[5,23-25]. Recently, an unique intestinal microbiota signature has been identified in an animal model of RP, which seems to be capable of increasing the expression of IL-1β, IL-6, and tumour necrosis factor-α (Figure 2). Mice colonised with previously irradiated intestinal microbiota were more prone to RP than the control colonised with naïve intestinal microbiota[26]. Furthermore, it is postulated that the microbiota can also influence the efficacy of the cancer treatment *per se*.

This transmural pathological regenerative process of the intestinal wall will exacerbate the progressive decrease in vascular density (and consequently the formation of friable telangiectasias and lymphatic vessels dilation), cell depletion, mucosal atrophy, and stenosis of the intestinal wall. Moreover, chronic mucosal ulcers can predispose to haemorrhage, the formation of fistulae and possible risk of intestinal perforation (Figure 2)[6,8,9].

CLINICAL PRESENTATION

Clinically, RP presents itself in two forms: acute or chronic. The acute form is typically resolved within a few weeks and it is characterised by nausea, vomiting, abdominal pain, diarrhoea, urgency, tenesmus, and more rarely, lower gastrointestinal bleeding. The chronic form has a similar clinical presentation; however, it is characterised by an indelible evolution, leading, in the most severe cases, to major digestive haemorrhage, chronic constipation, faecal incontinence, severe proctalgia, stenosis, fistulisation and eventually, intestinal perforation. Moreover, patients may also have other symptoms resulting from pelvic irradiation such as radiation enteritis, haemorrhagic radiation cystitis or urethral stricture. The occurrence of colorectal neoplasms induced by radiation has also been described, mostly after a long latency period, which can be manifested by masses or non-healing ulcers[5,7,8].

For diagnosis, a cause-effect relationship should be established between the history of pelvic RT and intestinal symptoms. A rectal examination will evaluate anal sphincter tonicity and a rectosigmoidoscopy the mucosa characteristics and affected areas, excluding malignancy. Total colonoscopy can be considered to outline the true extent of the disease and/or exclude the possibility of another aetiology for colitis. Endoscopic images usually reveal a pale, friable mucosa, with telangiectasias and/or ulcerations, and a clear separation between the altered and normal region, corresponding to the irradiated zone's limit. A biopsy is usually not recommended to confirm the diagnosis because it may increase the risk of complications[7,8].

In addition, it is essential to exclude other possible causes of subacute or chronic proctitis such as inflammatory bowel disease, diverticular colitis, atherosclerotic disease or previous episodes of chronic ischemic colitis, chronic exposure to the effects of non-steroidal anti-inflammatory drugs, recent use of antibiotics that predispose to *Clostridioides difficile* infection, parasitic (*e.g.*, amebiasis) or bacterial (*e.g.*, *Salmonella* spp., *Campylobacter* spp.) infections due to recent travelling to endemic countries, history or risk factors for sexually transmitted diseases (*e.g.*, *Neisseria gonorrhoeae* and herpes simplex virus) and cytomegalovirus infection in the immunocompromised patient[7,8].

In several studies, the severity of RP is objectively graded using symptom scores, such as the Radiation Proctopathy System Assessment Scale or the Late Effects Normal Tissue (LENT-SOMA) scale and considering the intraluminal findings by endoscopic grade (modified Chi grading or scales Chutkan and Gilinski). The comparison of data between studies is difficult due to the use of different severity scores. The same is true for outcome measures. Table 1 summarises some of the different classifications of RP that have been proposed in the literature[27-37].

Table 1 Classification of stages/grades of radiation proctitis

Ref.	Stages/grades	Description
Sherman[27], 1954	I-IV	Based on endoscopic findings: I: (a) Localised erythema and telangiectasia, friable mucosa with easy bleeding: no ulceration or stricture formation, and (b) More diffuse erythema along with periproctitis, marked pain, and sensitivity; II: Presence of ulceration with a greyish tenacious slough, usually involving the anterior rectal wall, and proctitis with grade I lesions; III: Presence of rectovaginal fistulae or bowel perforation and varying degrees of proctitis with ulceration; IV: Presence of rectovaginal fistulae or bowel perforation and varying degrees of proctitis with ulceration.
Dean and Taylor[28], 1960	I-III	Based on clinical and endoscopic findings: I: Symptoms: Rectal bleeding, tenesmus, sphincter instability, mucoid discharge; endoscopic findings of vascular congestion, friability of the mucosa, mucosal thickening, mucoid discharge; II: Same symptoms as before; endoscopic findings of ulcerations, underlying thrombosis of the small vessels; III: Same symptoms as before plus perineal sepsis, incontinence, diarrhoea, perianal purulent discharge; endoscopic findings of necrosis, fistulae, strictures.
Gilinsky *et al*[29], 1983	Normal; Mild; Moderate; Severe	Based on endoscopic findings: Score 0: Normal mucosa; Score 3: Erythema and/or telangiectasia, oedema, thickening, pallor of mucosa; Score 6: Friability; Score 9: Ulceration and/or necrosis.
Langberg *et al*[30], 1992	1-3	Based on histopathologic findings: Thickening of serosa: (1) Slight thickening of serosa, hyperplasia of peritoneal mesothelium; and (2) Marked thickening of serosa; and (3) Extreme thickening and fibrosis serosa. Mucosal alterations: (1) Small superficial ulcerations; and (2) Ulcerations involving more than half of the intestinal circumference. Epithelial atypia: (1) Abnormally oriented crypts; (2) Irregular crypt regeneration with atypical epithelial cells; and (3) Adenocarcinoma. Vascular sclerosis: (1) Slight double normal thickness, broadened and hyalinised collagen fibres; (2) Submucosa three to four times normal thickness, abnormal collagen fibres; and (3) Massive fibrosis including muscularis. Lymph congestion: (1) Dilated lymph vessels or cystic collections of lymph. Ileitis cystica profunda: (1) Submucosal glandular inclusions; (2) Submucosal cysts with polypoid elevation of the mucosa; and (3) Large cysts extending into the muscularis.
Chutkan *et al*[31], 1997	0-4	Based on clinical findings: 0: No haemorrhage; 1: Blood on toilet paper or mixed with faeces; 2: Drops of blood in the toilet; 3: Severe haemorrhage with expulsion of clots; 4: Haemorrhage that requires transfusion.
Wachter *et al*[32], 2000. Vienna Rectoscopy Score	0-5	Based on endoscopic findings: Score 0: Congested mucosa (grade 1); Score 1: Congested mucosa (grade 2), telangiectasia (grade 1); Score 2: Congested mucosa (grade 3), telangiectasia (grade 2); Score 3: Congested mucosa (any grade), telangiectasia (grade 3), ulceration (grade 1); Score 4: Congested mucosa (any grade), telangiectasia (any grade), ulceration (grade 2), stricture (grade 1); Score 5: Congested mucosa (any grade), telangiectasia (any grade), ulceration (grade 3), stricture (grade 2), necrosis (any grade).
Zinicola *et al*[33], 2003. Bleeding Scale for Radiation-Induced Haemorrhagic Proctitis	0-4	Based on clinical findings: 0: No bleeding; 1: Intermittent bleeding (once weekly or less); 2: Persistent bleeding (twice or more weekly); 3: Daily bleeding or anaemia; 4: Require blood transfusion.
Chi *et al*[34], 2005. RTD grading scale	0-3	Based on RTD endoscopic findings: 0: Normal mucosa; 1: < 10 telangiectasias; 2: > 10 telangiectasias; 3: Confluent lesions, active bleeding or friable mucosa.
Ehrenpreis *et al*[35], 2005. Radiation Proctopathy System Assessment Scale (RPSAS)	1-5	Based on clinical findings: Diarrhoea. Urgency. Rectal pain. Tenesmus. Rectal bleeding. Faecal incontinence. Severity: 1: No problem. 2: Mild problem–can be ignored when you do not think about it. 3: Moderate problem–cannot be ignored; no effect on ADL. 4: Severe problem–influences your concentration on ADL. 5: Very severe problem–markedly influences your ADL and/or requires rest. Frequency: 1: Monthly; 2: Weekly; 3: Several times *per* week; 4: Daily; 5: Throughout the day.
Cox *et al*[36], 1995. Late Radiation Morbidity Scoring Criteria Radiation Therapy Oncology Group/European Organisation for Research and Treatment of Cancer	0-5. Late (> 3 mo)	Based on clinical and imaging findings: 0: No changes; 1: Mild diarrhoea, mild cramping, bowel movement 5 times daily, slight rectal discharge or bleeding; 2: Moderate diarrhoea or colic, bowel movement > 5 times daily, excessive rectal mucus or intermittent bleeding; 3: Obstruction or bleeding requiring surgery; 4: Necrosis, perforation, or fistulae; 5: Death related to adverse event.
NCI CTCAE version 5.0[37], 2017	1-5	Based on clinical findings: 1: Mild adverse event; rectal discomfort, intervention not indicated; 2: Moderate adverse event; rectal discomfort, passing blood or mucus, medical intervention indicated, limiting instrumental ADL; 3: Severe and undesirable adverse event, faecal urgency or stool incontinence, limiting self-care ADL; 4: Life-threatening or disabling adverse event, urgent intervention needed; 5: Death related to adverse event.

ADL: Activities of daily living; RTD: Rectal telangiectasia density.

RP TREATMENT

The treatment of acute RP is essentially symptomatic and in accordance with the guidelines for the treatment of mucositis of other aetiologies[38,39]. In the absence of response to first-line antidiarrheal medication, it must be recommended treatment with octreotide or other somatostatin analogues and butyrate enemas that seem to accelerate the intestinal mucosa regeneration process. There is clinical evidence that

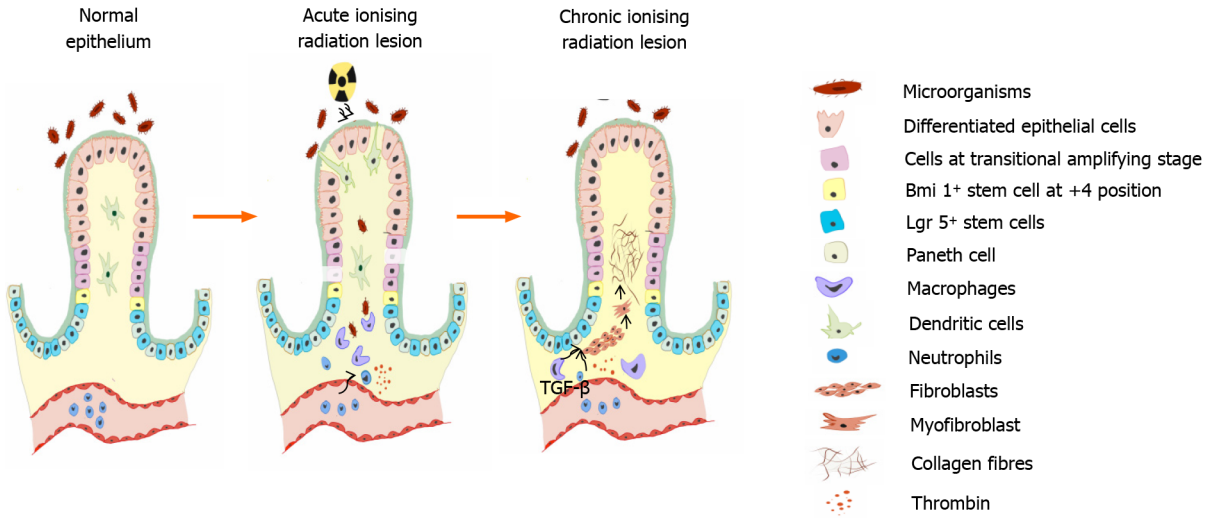


Figure 1 Representative scheme of intestinal injury induced by ionising radiation. In a healthy gut, crypts are with intact mucosa. Lgr5$^+$ stem cells proliferate and cells migrate upwards to provide differentiated epithelial cells of the villi. Acute lesions occur predominantly through different pathological processes: Depletion of epithelial cells due to cytotoxicity in progenitor cells and consequent apoptosis; inflammation and infiltration of the lamina propria with polymorphonuclear leukocytes and plasma cells; eosinophilic abscesses of intestinal crypts; endothelial lesions of intestinal microvascularisation with the release of thrombin and eventual oedema of the submucosa; influx of antigenic material, including gut microbiota into the lamina propria. If the submucosa modifications are not impactful, the epithelial cells regenerate, and the process resolves spontaneously. The constitutive and chronic phase comprises obliterating endarteritis with progressive reduction of parietal irrigation and consequent local ischaemia; formation and diffuse progression of mucosal and submucosal fibrosis through a local proinflammatory cytokine cascade (high levels of interleukin-1β (IL-1β), IL-2, IL-6, IL-8, and transforming growth factor-β), which is promoted by macrophages, neutrophils and by the differentiation of fibroblasts into myofibroblasts. Citation: Adapted from Costa *et al*[21] and Kumagai *et al*[23].

intrarectal amifostine (ROS scavenger) could be, in selected patients, a possible protective pharmacological measure against the acute effects of RT. Generally, the acute RP is a self-limiting situation, although in about 20% of cases, it is necessary to suspend or even interrupt RT treatments[7,38,39].

The natural history of RP *per se* is unpredictable. Minor symptoms may resolve spontaneously or require conservative treatment. On the other hand, symptoms may persist for similar and uncomplicated clinical contexts and become refractory to the progressive increase in therapeutic intervention. Thus, early diagnosis and intervention are essential and symptoms should not be overlooked, even if they have little impact on the patient's quality of life (QoL).

The management of RP can be challenging and requires a holistic approach for which there is no gold standard protocol. Furthermore, there are no prophylactic measures that have been shown to be beneficial in reducing RP incidence. Concerning treatment options, most of the clinical evidence is based on case studies and small series. Therapeutic management should be personalised according to the patient, the severity of the clinical condition, and the institution's experience. The non-invasive or minimally invasive measures include changing the diet and symptom control with pharmacological and endoscopic support. In terms of medical treatment, the intrarectal application of formalin (4%-10% formaldehyde) and sucralfate enemas stand out as effective measures to control bleeding in patients with haemorrhagic RP. Studies present conflicting results for the use of short-chain fatty acid enemas. There is a lack of scientific evidence to recommend the use of alternative treatments such as mesalamine, ozone therapy, metronidazole, vitamin A, antioxidant vitamin complexes (vitamin C and E) and pentoxifylline. Endoscopic treatment with plasma argon has been effective in controlling and treating lower gastrointestinal bleeding. Endoscopic bipolar electrocoagulation, radiofrequency ablation, Nd: YAG laser and cryotherapy are possible alternatives to argon, although their evidence level is low. Despite the enormous therapeutic armamentarium, the more severe RP cases may require radiological intervention for lower gastrointestinal bleeding and surgical intervention, especially in complications such as effective control of lower gastrointestinal bleeding, fistulisation, occlusion, and intestinal perforation[3,5,7,8,38,39].

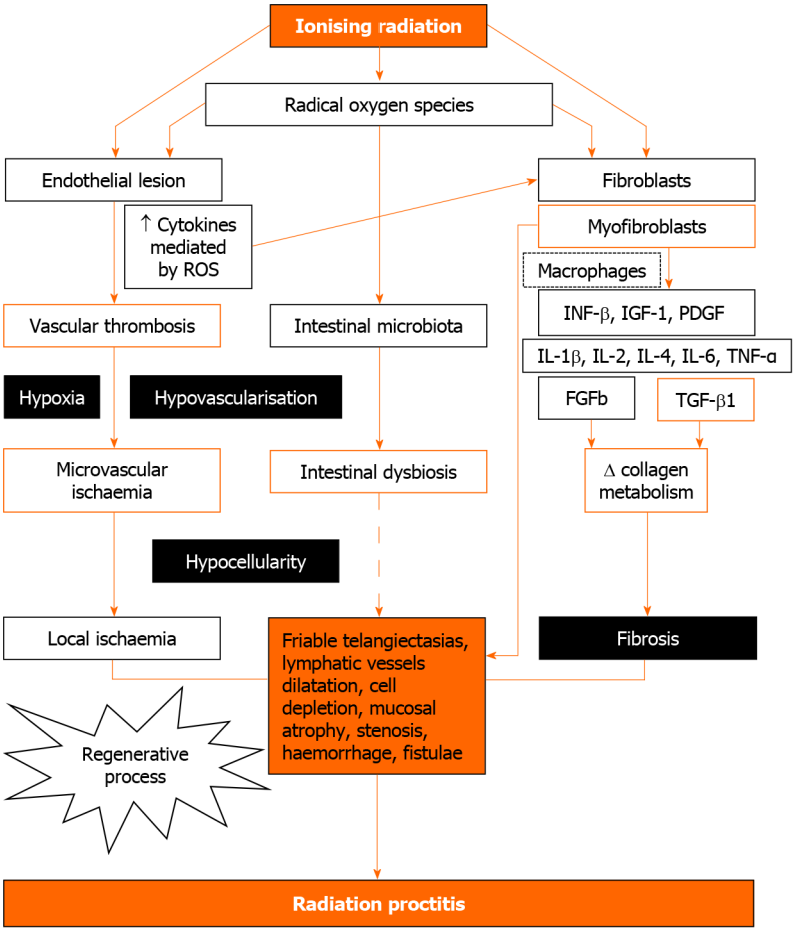


Figure 2 Representative scheme of the several pathophysiological mechanisms involved in radiation proctitis: The hypoxia/hypocellularity/hypovascularisation, intestinal microbiota and the fibroatrophic theories. FGFb: Basic fibroblast growth factor; IGF1: Insulin-like growth factor 1; IL: Interleukin; INF-β: Interferon- beta; PDGF: Platelet-derived growth factor; ROS: Reactive oxygen species; TGF-β1: Transforming growth factor beta-1; TNF-α: Tumour necrosis factor-alpha. Citation: Adapted from Costa DA *et al*[21].

HBOT

HBOT is a treatment based on the inhalation of pure oxygen (100%) in an environment with an atmospheric pressure higher than that existing at sea level (1 atmosphere absolute [ATA]). The HBOT sessions are held inside hermetically sealed hyperbaric chambers, classified as type IIb medical devices (directive 93/42 ECC of June 14, 1993, concerning medical devices). HBOT is used in several clinical conditions as well as in professional and military training. Therapeutic HBOT usually involves pressures higher than 1.4 ATA (141.8 kPa), most frequently ranging between 2.0 (202.6 kPa) and 2.5 ATA (253.3 kPa) for 60 to 120 min[40].

Currently, the ECHM recommends HBOT for the treatment of RP (degree of recommendation I/ level of evidence B)[22]. Unlike most conventional treatment that only alleviated symptoms, HBOT can favourably change the natural history of other RT late sequelae[4]. Its clinical benefit emanates from the therapeutic effects of hyperbaric oxygen that include, among others, the promotion of tissue oxygenation, neovascularisation, reepithelialisation, and the reversal of the fibroatrophic process induced by ionising radiation[16,21,41,42].

The mechanisms that result in HBOT beneficial effects can also cause side effects in some patients, primarily due to pressure and oxygen toxicity. However, when appropriate therapeutics protocols are applied, HBOT is a safe and low-risk intervention, with the adverse events being infrequent and typically not severe[40-43].

In 1991, Charneau *et al*[44] treated the first RP patient with HBOT. The 74-year-old patient had a 5-mo history of transfusion-dependent haemorrhagic RP. For this patient, after the failure of previous treatments (enema with corticosteroids and Nd: YAG laser), HBOT was considered a strategy to avoid surgery. After 82 sessions of HBOT (2.5 ATA, for 90 min, twice daily), a complete clinical response was observed, which remained during the follow-up period of at least 9 mo. After this pioneering

report, others were published, including case reports, retrospective, (non)-randomised studies and clinical trials, as well as several reviews and meta-analyses that studied the clinical impact of HBOT on RP (Table 2)[16,45-71]. However, most studies had both a small number of cases treated and a short follow-up period.

In 1997, Warren *et al*[47] conducted a study with 14 patients undergoing two pressurisation regimens (n = 9; 2 ATA, for 120 min, 5-6 times per week; n = 5; 2.35 ATA, for 90 min, 5 times per week). The authors documented an overall response rate of 64.3% (57.1% with complete responses) for an average follow-up period of 14.6 (range 2-35) mo. In the same year, Woo *et al*[48], published a study with 18 patients submitted to 24 sessions of HBOT (2 ATA, for 105 min, 6 times per week) with a follow-up period of 14 (range 3-65) mo. In this study, different response rates were reported according to the symptoms analysed: Haemorrhage 41.2% (7/17); rectal pain 50% (2/4); incontinence 75% (3/4) and diarrhoea 50% (4/8). Furthermore, most patients (77.7%) had already undergone other treatments but with little clinical benefit (n = 13, enema with corticosteroids; n = 1, formalin). Gouëllo *et al*[52], published a study with 36 patients submitted to an average of 67 sessions of HBOT (2.5 ATA, for 90 min, 5 times per week). An overall response rate of 53% (19/36) was observed immediately after the end of treatment. In the 52-mo follow-up period, the overall response rate was 66% (21/32). In 2002, in a systematic review published by Feldmeier and Hampson[2], 14 publications were evaluated in the context of RP and radiation enteritis (12 studies in humans and 2 in animals). Of the 9 studies, 114 patients were considered, and an overall response rate of 95.6% was documented (36%, 41 patients with complete response; 60%, 68 patients with better symptomatic control). Despite these studies have shown an improvement in RP with HBOT, a clinical benefit has also been verified in the small intestine radio-induced malabsorption syndrome. In 2007, Marshall *et al*[16], published the largest study carried out up to that time, with 65 patients with enteritis and RP (85% of patients). The authors described an overall response rate of 68% (43% with complete responses) after at least 30 sessions of HBOT (2.4 ATA, for 90 min, 5 times per week). However, half of the patients (49.2%, 32) had to be submitted to more HBOT sessions, up to a maximum of 60, due to partial response or recurrence of symptoms. The mean follow-up time was 23 (range 1-70) mo. The response rate of patients with low gastrointestinal bleeding was 70%. In those dependent on transfusions, the response rate was very satisfactory since 75% of these patients no longer needed transfusion support. For symptoms other than haemorrhage, the response rate was 58%, with pain reduction, nutritional status improvement, intestinal transit regularisation and even fistulae closure. Moreover, no correlation was established between the treatment response rate and the duration of symptoms or the time between RT and initiation of HBOT.

In 2008, Clarke *et al*[65] published the results of the first multicentre, randomised, sham-controlled, double-blind clinical trial (HORTIS) that included patients with RP refractory to other therapeutic interventions. A total of 150 patients were enrolled, but only 120 completed the study. Patients were randomised to HBOT (Group 1: 2.0 ATA, for 90 min, 5 times per week) or sham treatment with 21% oxygen (Group 2: 1.34-> 1.1 ATA, for 90 min, 5 times per week). The clinical response evaluation was performed after 30 treatment sessions, with the possibility of 10 additional sessions, depending on the investigator's decision. Only 3 patients did not accept the crossover to the active arm of the initial HBOT (Group 1). After adjusting covariates and for an average follow-up period of 2 years, Group 1 significantly improved the mean LENT-SOMA score (5.00 *vs* 2.61, P = 0.0019). In the initial allocation phase and after the first efficacy assessment, the experimental group's overall response rate was higher than in the control group (88.9% *vs* 62.5%, P = 0.0009). Furthermore, there was also a significant improvement in QoL (pattern of pain, bleeding and intestinal transit) in Group 1 (including in the crossover subgroup of patients). It is noteworthy that, for Group 1, the improvement in QoL in terms of faecal incontinence, faecal urgency, pain and intestinal transit was consistent throughout the follow-up period. In 2015, Tahir *et al*[69] reported an overall response rate of 95% in the 59 patients treated with RP (51% of patients with complete response for a median duration of 15 mo). Bennett *et al*[72], in a Cochrane meta-analysis published in 2016, documented a significant increase in the clinical improvement or even remission of RP after HBOT (relative risk 1.72; 95% confidence interval 1.0-2.9, P = 0.04, NNT to benefit 5).

In 2016, Glover *et al*[70], in a multicentre, randomised, double-blind, sham-controlled phase 3 trial (HOT2), evaluated the clinical benefit of HBOT in patients with chronic bowel dysfunction after RT in the context of pelvic neoplasms. Treatment efficacy was determined by comparing the Inflammatory Bowel Disease Questionnaire (IBDQ) and the IBDQ rectal bleeding scores assessed before and 12 mo after starting treatment. Moreover, other secondary endpoints were evaluated: LENT-SOMA, the

Table 2 Summary of the case-series or clinical trials described in the literature regarding the use of hyperbaric oxygen therapy in the treatment of radiation proctitis

Ref.	Study design	Patients (*n*)	RP stages/grades, patients (*n*)	Other (previous) treatments (%)	HBOT protocol	Nº sessions	Overall response rate (%)	Complete response (%)	Follow-up period (mo)
Bouachour *et al*[45], 1990	Retrospective	8	NA	100%	2.5 ATA, 90 min, twice daily (4 wk)-> Once daily	80 ± 10	87.5%	75%	[4-20]
Feldmeier *et al*[46], 1996	Retrospective	7	RTOG/EORTC[36]: 4	28.6%	2.4 ATA, 90 min, 5 ×/wk	24 [3-50]	57%	57%	NA
Warren *et al*[47], 1997	Retrospective	14	Bleeding (*n* = 11); Diarrhoea (*n* = 5); Rectal pain (*n* = 4); Tenesmus (*n* = 2)	78.6%	2.0 ATA, 120 min, 5-6/wk; 2.35 ATA, 90 min, 5 ×/wk	39 [20-72]	64.3%	57.1%	14.6 [2-35]
Woo *et al* [48], 1997	Retrospective	18	Bleeding (*n* = 17); Diarrhoea (*n* = 8); Rectal pain (*n* = 4); Incontinence (*n* = 4)	77.7%	2.0 ATA, 105 min, 6 ×/wk	24 [40]	55.5%; Incontinence 75%; Diarrhoea 50%; Rectal pain 50%; Haemorrhage 41.2%	11.1%; 50%; 25%; 25%; 23.5%	14 [65]
Bredfeldt and Hampson [49], 1998	Retrospective	19	NA	NA	2.36 ATA, 90 min, 5 ×/wk	30	84%	47%	NA
Ugheoke *et al*[50], 1998	Retrospective	8	NA	NA	2.5 ATA, 90 min, 5 ×/wk	28 [20-40]	62.5%	NA	NA
Carl *et al* [51], 1998	Retrospective	2	Bleeding (*n* = 1); Rectal pain (*n* = 1)	NA	2.4 ATA, 90 min, 5 ×/wk	38, 40	50%	50%	NA
Gouëllo *et al*[52], 1999	Retrospective	36	LENT-SOMA: Grade 1 (*n* = 1); Grade 2 (*n* = 11); Grade 3 (*n* = 16); Grade 4 (*n* = 8)	NA	2.5 ATA, 90 min, 5 ×/wk	67 (mean)	66%	25%	52
Kitta *et al* [53], 2000	Retrospective	4	Bleeding (*n* = 3); Rectal pain (*n* = 1)	100%	2.0 ATA, 60 min, 5 ×/wk	37.5 [30-60]	75%	25%	NA
Bem *et al* [54], 2000	Retrospective	2	Dean and Taylor [28]: I-II	100%	2.4 ATA, 90 min, 5 ×/wk	60, 60	100%	100%	[3-48]
Roque *et al* [55], 2001	Retrospective	6	NA	NA	2.5 ATA, 90 min, 5 ×/wk	37 [20-60]	85%	NA	NA
Mayer *et al* [56], 2001	Retrospective	10	RTOG/EORTC[36]: Grade 2 (*n* = 4); Grade 3 (*n* = 6)	Majority (% not stated)	2.2-2.4 ATA, 60 min, 7 ×/wk	28 [13-60]	90%	30%	15.3 [7.5-26.9]
Boyle *et al* [57], 2002	Retrospective	19	NA	NA	2.0 ATA, 120 min, 5 ×/wk	59 [27-80]	68%	NA	NA
Jones *et al* [58], 2006	Retrospective	10	LENT-SOMA: Grade 2 (*n* = 7); Grade 3 (*n* = 3)	100%	2.0-2.5, 90 min, 5 ×/wk	40 [36-41]	77%; Haemorrhage 88.8%; Diarrhoea 80%; Rectal pain 80%	44.4%; 60%; 20%	25 [6-43]
Dall'Era *et al*[59], 2006	Retrospective	27	RTOG/EORTC[36]: 3-4	100%	2.4 ATA, 90 min, 7-7 ×/wk	36 [29-60]	66.6%; Haemorrhage 76%; Rectal pain 75%; Faecal urgency 75%; Rectal ulcer 50%	37%; 48%; 0%; 50%; 21%	13 [1-60]
Fink *et al* [60], 2006	Retrospective	4	NA	100%	2.4 ATA, 90 min, 5 ×/wk	31 [28-37]	75%	25%	33
Girnius *et al*[61], 2006	Retrospective	9	Bleeding Scale for Radiation-Induced	100%	2.5 ATA, 90 min, 5 ×/wk	58 [22-80	100%	77.7%	17 [1-77]

			Haemorrhagic Proctitis[33]: Grade 2 (n = 1); Grade 3 (n = 3); Grade 4 (n = 5)						
Marshall *et al*[16], 2007	Retrospective	65 (15 with lesions beyond the rectum)	Bleeding (n = 54); Diarrhoea (n = 25); Rectal pain (n = 25); Tenesmus, urgency, incontinence (n = 13); Malnutrition, weight loss (n = 7); Bloating, cramping (n = 6); Nausea, emesis (n = 5); Fistulae (n = 2); Total parenteral nutrition (n = 2)	65%	2.36 ATA, 90 min, 5 ×/wk	30-> If partial response-> 60	68% (all patients); 65% (rectum); 73% (proximal sites); Haemorrhage 70%; Other symptoms 58% (including pain relief, improved nutritional status and intestinal transit, closure of fistulae)	43%; 39%; 60%; 75%; 33%	23 [1-70]
Sidik *et al* [62,63], 2007	Prospective, randomised clinical trial	HBOT 32; Comparator33	LENT-SOMA: HBOT, mean 7.7 ± 2.0; Control, mean 6.8 ± 2.3. Karnofsky scale: HBOT, mean 73.8 ± 6; Control, mean 74.6 ± 8.3	NA	HBOT, Protocol not reported *vs* Comparator described as "symptomatic treatment"	HBOT, Minimum 18 sessions	Outcomes poorly reported (losses to follow-up, not all patient data provided)	NA	6
Safra *et al* [64], 2008	Retrospective	6	NCI CTCAE[37]: Mean 3.3 [2-4]	100%	2.0 ATA, 90 min, 7 ×/wk	27 [16-40]. Not only RP	100%	16.7%	NA
Clarke *et al* [65], 2008	Randomised, double-blind, sham-controlled, crossover allowed ("HORTIS")	HBOT 76; Comparator74	LENT-SOMA: HBOT, mean 12.55; Sham, mean 12.84	100%	HBOT, 2.0 ATA, 90 min, 5 ×/wk *vs* Sham treatment 1.34-> 1.1 ATA O_2 21%, 90 min, 5 ×/wk	30-> 40	HBOT: 88.9%. Improved bowel-specific and bowel bother and function QoL scores (before crossover) *vs* Sham treatment: 62.5%	HBOT: 7.9% *vs* Sham: 0%	25 [12-60]
Alvaro-Villegas *et al*[66], 2011	Prospective, non-randomised clinical trial	HBOT 17; Comparator14	LENT-SOMA: HBOT, 12.1 ± 2.9; APC, 13.3 ± 2.9	NA	HBOT 2.0-2.5 ATA, 90 min, 5 ×/wk *vs* Non-contact APC, 2.3 mm diameter catheter, 1.6 L/min flow rate at 60 W, mean 3 ± 1 (SD) sessions	HBOT, 35 ± 5 *vs* APC, 3 ± 1	HBOT: 82% *vs* APC: 87%	NA	3
Oliai *et al* [67], 2012	Retrospective	4	LENT-SOMA: Mean 0.66 [0.36-0.93]. Severity of rectal bleeding: Persistent (n = 3), Occasional (n = 1)	100%	2.0 ATA, 90-105 min, 5 ×/wk	37.5 [30-40]	75%	50%	NA
Carvalho *et al*[68], 2014	Retrospective	30	NA	NA	2.5 ATA, 100 min, 5 ×/wk	66 [38-80]	96.7%	73.3%	NA
Tahir *et al* [69], 2015	Retrospective	59	NA	NA	2.4 ATA, 70 min, 7x/wk	NA	95%	51%	Major response 15 [2-76]. Minor response 20 [1-84]
Glover *et al*[70], 2016	Randomised, double-blind, sham-controlled phase 3 clinical trial ("HOT2")	Ratio 2:1; HBOT 55; Comparator29	LENT-SOMA: Grade 2; Grade 1 with intermittent symptoms. IBDQ bowel function component (n/IQR): HBOT 48 (42-52); Sham 51 (44-59). IBDQ rectal	100%	HBOT 2.4 ATA, 90 min, 5 ×/wk *vs* Sham treatment 1.34 ATA O_2 21%, 90 min, 5 ×/wk	40 (89% of patients) < 38 (11% of patients)	HBOT: IBDQ bowel function component (n/IQR): Δ 3.5 (-3-11). IBDQ rectal bleeding (n/IQR): Δ 3 (1-3) *vs* Sham treatment: IBDQ	NA	13.2 [12.4-14.2]

			bleeding (n/IQR): HBOT 3 (2-4); Sham 3 (2-4). NCI CTCAE [64]: Grade 1-3, 46 (55%). EORTC QLQ-CR38 Question 59: Grade 1-3, 47 (59%)				bowel function component (n/IQR): Δ 4 (-6-9); IBDQ rectal bleeding (n/IQR): Δ 1 (1-2)		
Yoshimizu *et al*[71], 2017	Retrospective	5	Sherman[27]: Grade II (*n* = 3); Grade III (*n* = 1); Grade IV (*n* = 1)	NA	2.5 ATA, 60 min, 5 ×/wk	76 [40-100]	100%	20%	NA

APC: Argon plasma coagulation; ATA: Atmosphere absolute; HBOT: Hyperbaric oxygen therapy; IBDQ: Inflammatory Bowel Disease Questionnaire; IQR: Interquartile range; min, minute; NA: Not available or not applicable; NCI CTCAE: National Cancer Institute Common Terminology Criteria for Adverse Events; O_2: Oxygen; QoL: Quality of life; RP: Radiation proctitis; SD: Standard deviation; Δ: Median change from baseline to 12 mo.

11-question questionnaire related to symptoms selected from the CTCAE gastrointestinal scale (version 4.0) and QoL through validated questionnaires (Questionnaire basic EORTC QLQ-C30 and colorectal module QLQ-CR38). Patients of both sexes with ages over 18 years were included, with at least grade 2 gastrointestinal symptoms of any category of LENT-SOMA or grade 1 with intermittent symptoms due to RT performed at least 12 mo before in the context of rectal, prostate, testicle, bladder, cervix, vagina, vulva or ovary neoplasms. Patients with grade 3 symptoms LENT-SOMA were excluded since they have higher affectation on activities of daily living and require, generally, more aggressive treatments. Patients with RT symptoms were considered eligible for the trial only if they had been submitted to other general interventions for at least 3 mo (*e.g.*, diet change, oral therapeutic optimisation) with no improvement. Patients were randomised in a 2:1 ratio in favour of HBOT and stratified by centre. Patients were randomised to HBOT (2.4 ATA, for 90 min, 5 times per week) or 21% oxygen (1.3 ATA, for 90 min, 5 times per week). Of the 84 patients included in this clinical trial, 55 underwent HBOT and 29 sham treatment. In the analysis of clinical efficacy, no difference was identified between the two arms, when comparing the main endpoints: IBDQ bowel component [HBOT = 46; sham treatment = 23; HBOT: 4 (-3 to 11) *vs* sham treatment: 4 (-6 to 9), $P = 0.50$] and IBDQ rectal bleeding [HBOT = 29; sham treatment = 11; HBOT: 3 (1 to 3) *vs* sham treatment: 1 (1 to 2), $P = 0.092$].

The results of the HOT2 clinical trial are inconsistent with most previous clinical evidence, including the Cochrane meta-analysis[72], primarily based on the HORTIS [65] clinical trial. The HOT2 study[70] is a very relevant clinical trial from a methodological point of view: Phase 3, randomisation 2:1, stratification by centre, double-blind, sham-controlled, with primary and secondary endpoints with previously validated scores and questionnaires and with well-defined inclusion/exclusion criteria, including a period of at least 3 mo for possible symptom optimisation and control. However, one can enumerate some aspects that can be criticised (not only exclusively for this trial): (1) The sample size is debatable for a phase 3 clinical trial when it is supposed to comprises a total number of patients over 300. The statistical calculation was based on assumptions defined and validated in previous studies. However, the comparison between the two arms was performed with an insufficient number of patients (HBOT: 46 *vs* sham treatment: 23); (2) The drop-out rate was 17.86% (15 patients: 9 from the HBOT group and 6 from sham treatment), with only 69 out of 84 patients having a 12-mo follow-up period. The final statistical power was 75%, although the study had been designed for 80% power; (3) Patients with symptoms ≥ grade 3 LENT-SOMA (*e.g.*, severe faecal incontinence or transfusion-dependent RP) were not included. Thus, one cannot generalise the results for this subgroup; (4) It is unknown whether the percentage of patients with a medical history of lower gastrointestinal bleeding influences their progress during the study (HBOT: 62% *vs* sham treatment: 79%); (5) The dietary and/or pharmacological measures performed in the period prior to randomisation were not discriminated (*e.g.*, diet type, probiotics, antibiotic cycles). Did these different procedures influence the natural history of the disease and the potential response to treatment? (6) The smoking habits of the patients were not quantified. Otto *et al*[73], in a study on diabetic foot, determined that patients with above a 10 pack-year (P-Y) history of smoking would need an average of more than 8 to 14 sessions of HBOT to obtain the same therapeutic effect as non-smokers. Freiberger *et al*[74] reported that in patients with mandible osteoradionecrosis, those who smoked showed a shorter maintained treatment period response to HBOT when compared to non-smoking patients (15.8 *vs* 86.1 mo). The stratification by smoking

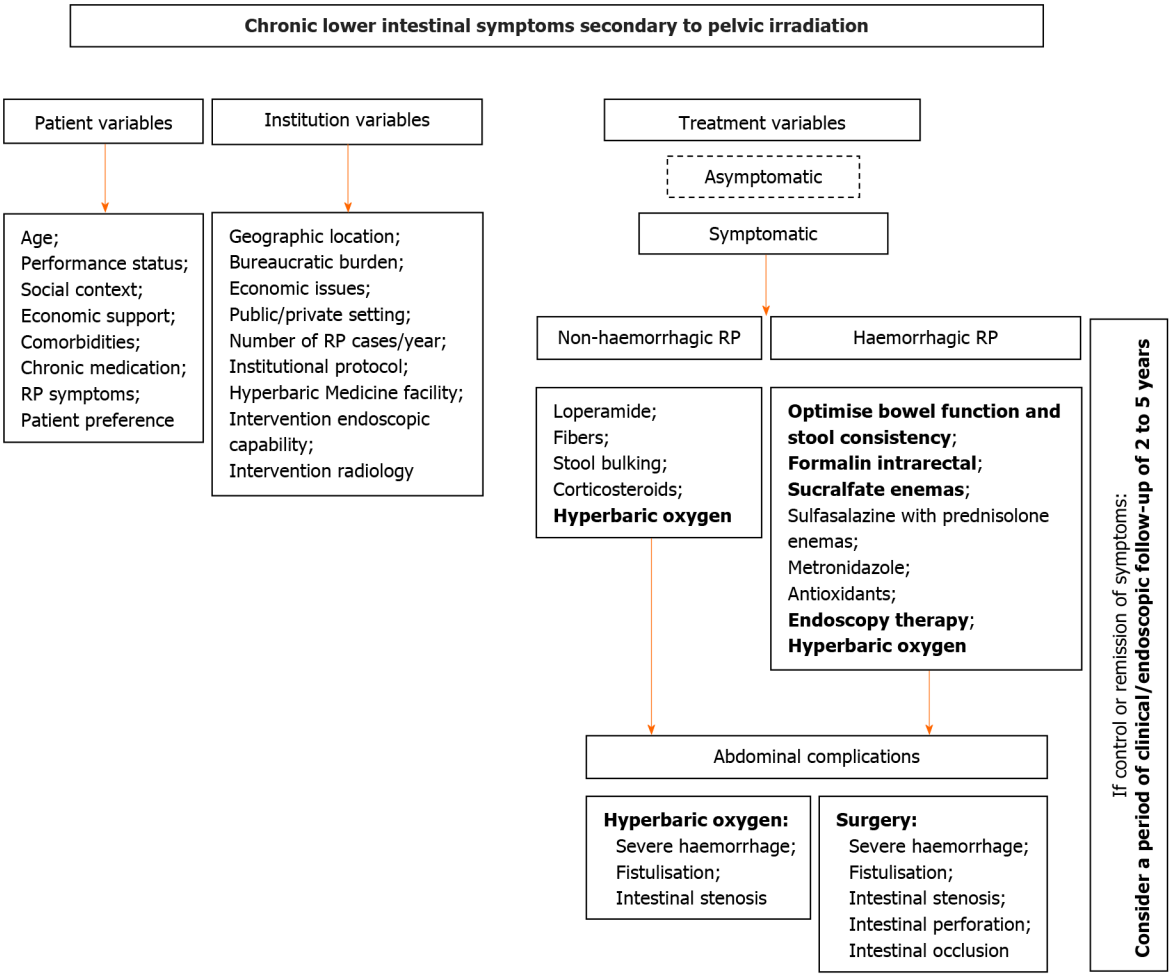


Figure 3 Suggested treatment algorithm for radiation proctitis based on variables related to the patient, institution, and the severity of the clinical context. RP: Radiation proctitis; RT: Radiotherapy.

habits could have been relevant in this trial (non-smoker or smoker ≤10 P-Y *vs* >10 P-Y); (7) The distribution of the subgroup of patients (n = 9; 11%) who did not complete the 40 treatment sessions in both arms of the clinical trial is unknown; (8) The endoscopic response to the recommended treatments is unknown; (9) The clinical and endoscopic evolution beyond the median follow-up period 13.2 (range 12.4-14.2) is also unknown. In HORTIS clinical trial[65] it was observed and additional and maintained response beyond 12 mo of the follow-up period; and (10) The absence of a third arm in the study comparing HBOT to another conventional treatment for RP.

We recommend that HBOT, combined with nutritional support and local treatment, may be beneficial for patients with moderate to severe symptoms that do not require surgical intervention. The HBOT regimen should include at least 20-30 sessions with a pressure of 2 to 2.5 ATA for 60 to 120 min/d to ensure a more effective clinical response. The treatment protocol total duration may be extended to several weeks until a clinical and radiological complete response is obtained, and the follow-up should be personalised to each clinical context, considering a period of 2 to 5 years (Figure 3).

CONCLUSION

Although there are several effective therapeutic strategies to treat RP and improve its clinical condition, gold standard management has not yet been established. RP's management approach must be personalised according to the patient, clinical condition severity, and the institution's experience. The most conservative treatment comprises diet modulation and nutritional support, oral and intrarectal pharmacological treatment and HBOT. Endoscopic treatment may be indicated for the control and treatment of lower gastrointestinal bleeding. In severe refractory disease with

complications, surgical intervention should be considered.

During HBOT, the occurrence of adverse events is relatively infrequent. HBOT may potentially alleviate gastrointestinal radio-induced complications, including rectal bleeding, diarrhoea and pain. The authors' expectancies are that, in the near future, the controversy regarding HBOT in RP will be dimmed. More prospective and randomised studies are needed to validate the effectiveness of HBOT in the 'real-world' clinical practice.

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ORIGINAL ARTICLE

Retrospective Study

Multifocal autoimmune pancreatitis: A retrospective study in a single tertiary center of 26 patients with a 20-year literature review

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Author contributions: Huang XM did the imaging analysis and supervised all the procedures of this study; Shi ZS designed this study, wrote the main manuscript, revised the manuscript, and did statistical analysis; Shi ZS and Ma CL performed dynamic contrast enhanced-computed tomography scanning; Huang XM and Ma CL contributed equally.

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Conflict-of-interest statement:

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Abstract

BACKGROUND

Multifocal-type autoimmune pancreatitis (AIP), sometimes forming multiple pancreatic masses, is frequently misdiagnosed as pancreatic malignancy in routine clinical practice. It is critical to know the imaging features of multifocal-type AIP to prevent misdiagnosis and unnecessary surgery. To the best of our knowledge, there have been no studies evaluating the value of diffusionweighted imaging (DWI), axial fat-suppressed T1 weighted image (T1WI), and dynamic contrast enhanced-computed tomography (DCE-CT) in detecting the lesions of multifocal-type AIP.

AIM

To clarify the exact prevalence and radiological findings of multifocal AIP in our cohorts and compare the sensitivity of DWI, axial fat-suppressed T1WI, and DCE-CT for detecting AIP lesions. We also compared radiological features between multifocal AIP and pancreatic ductal adenocarcinoma with several key imaging landmarks.

METHODS

Twenty-six patients with proven multifocal AIP were retrospectively included. Two blinded independent radiologists rated their confidence level in detecting the lesions on a 5-point scale and assessed the diagnostic performance of DWI, axial fat-suppressed T1WI, and DCE-CT. CT and magnetic resonance imaging of multifocal AIP were systematically reviewed for typical imaging findings and compared with the key imaging features of pancreatic ductal adenocarcinoma.

RESULTS

Among 118 patients with AIP, 26 (22.0%) had multiple lesions (56 lesions).

There are no conflicts of interest related to this study.

Data sharing statement: No additional data are available.

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Ulcerative colitis was associated with multifocal AIP in 7.7% (2/26) of patients, and Crohn's disease was present in 15.3% (4/26) of patients. In multifocal AIP, multiple lesions, delayed homogeneous enhancement, multifocal strictures of the main pancreatic duct, capsule-like rim, lower apparent diffusion coefficient values, and elevated serum Ig4 level were observed significantly more frequently than pancreatic ductal adenocarcinoma, whereas the presence of capsule-like rim in multifocal-type AIP was lower in frequency than total AIP. Of these lesions of multifocal AIP, DWI detected 89.3% (50/56) and 82.1% (46/56) by the senior and junior radiologist, respectively.

CONCLUSION
Multifocal AIP is not as rare as previously thought and was seen in 22.0% of our patients. The diagnostic performance of DWI for detecting multifocal AIP was best followed by axial fat-suppressed T1WI and DCE-CT.

Key Words: Autoimmune pancreatitis; Magnetic resonance imaging; Diffusion-weighted magnetic resonance imaging; Multidetector computed tomography; Pancreatic ductal adenocarcinoma; Ulcerative colitis

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Core Tip: Multifocal autoimmune pancreatitis is not as rare as previously thought and was seen in 22.0% of our patients. The diagnostic performance of diffusionweighted imaging for detecting multifocal autoimmune pancreatitis was best followed by axial fat-suppressed T1 weighted image and dynamic contrast enhanced-computed tomography.

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INTRODUCTION

Autoimmune pancreatitis (AIP) is a distinctive type of chronic pancreatitis generally characterized by an autoimmune inflammatory condition in which prominent lymphoplasmacytic infiltration with associated pancreatic fibrosis causes organ dysfunction[1]. Previous studies have shown that AIP is recognized as a heterogeneous disease classified into at least two subtypes, including lymphoplasmacytic sclerosing pancreatitis (type 1) and idiopathic duct-centric pancreatitis (type 2) with granulocytic epithelial lesions[2]. Meanwhile, AIP has also been classified radiologically as diffuse, unifocal, or multifocal[3-5]. Diffuse-type AIP is easily distinguished from pancreatic ductal adenocarcinoma (PDA) by typical imaging features, *i.e.* diffuse sausage-like swelling of pancreatic parenchyma with capsule-like peripancreatic rim[6,7]. Moreover, some studies[4,8] recently attempted to identify differential imaging findings between unifocal AIP and PDA by using computed tomography (CT), magnetic resonance imaging (MRI), or magnetic resonance cholangiopancreatography. Several key imaging landmarks, with reference to radiological morphology of the pancreatic mass lesion and stricture pattern of the main pancreatic duct (MPD), were clearly identified to be crucial to distinguish unifocal AIP from PDA. Hence, the differential diagnosis between unifocal AIP and PDA may not be as challenging as before.

However, multifocal-type AIP, sometimes forming multiple pancreatic masses, is frequently misdiagnosed as pancreatic malignancy in routine clinical practice[9,10]. It is critical to know the imaging features of multifocal-type AIP to prevent misdiagnosis and unnecessary surgery. Consequently, a clearer diagnostic consensus on the imaging characteristics of multifocal AIP is needed to differentiate from pancreatic malignancy. For this purpose, we herein report 26 cases of multifocal AIP and a review of the previous literature with an emphasis on CT and MRI imaging features that may help

definitively distinguish between multifocal AIP and PDA.

CT is the most commonly performed, noninvasive modality to evaluate both pancreatic and extrapancreatic manifestations in patients with suspected AIP[4,11]. In our experience and according to the previous literature, CT has certain limitations in showing some specific findings of focal AIP, *i.e.*, focal masses and focal MPD strictures, while these imaging findings seem to be more clearly demonstrated on MRI, especially on diffusionweighted imaging (DWI)[12]. In our previous study, we confirmed that axial T1 weighted image (T1WI) is the optimal sequence of identifying homogenously isoattenuating insulinoma on dynamic contrast enhanced-computed tomography (DCE-CT)[13]. Zhu *et al*[14] found that the appearance of multiple solid lesions can provide a diagnostic clue for identifying pancreatic nonmalignancies. Therefore, it is essential to find an optimal modality to identify pancreatic lesions as correctly as possible. To the best of our knowledge, there have been no studies evaluating the value of DWI, axial fat-suppressed T1WI, and DCE-CT in detecting the lesions of multifocal-type AIP.

We aimed to clarify the exact prevalence and radiological findings of multifocal AIP in our cohorts and compare the sensitivity of DWI, axial fat-suppressed T1WI, and DCE-CT for detecting AIP lesions. We also compared radiological features between multifocal AIP and PDA with several key imaging landmarks.

MATERIALS AND METHODS

Study population

This retrospective study was approved by the Institutional Review Board of the First Affiliated Hospital of Fujian Medical University (Fuzhou, China), and written informed consent from patients was waived. The clinical, radiological, and pathological databases at our hospital between January 2005 and July 2020 were reviewed. A total of 234 consecutive patients who had undergone DCE-CT on multidetector computerized tomography and conventional MRI imaging protocols on a 3T scanner within 5 wk of each other for suspected AIP were included. Of these initially suspected cases, we finally identified 118 patients who were diagnosed with AIP according to international consensus diagnostic criteria[15].

Among these confirmed patients, 26 patients with 56 lesions were retrospectively included in our study, based on the following inclusion criteria: (1) the use of DCE-CT with the acquisition on the pancreatic arterial, pancreatic parenchyma, and portal venous phases; (2) conventional MRI sequences, including both axial fat-suppressed T1WI and DWI before steroid treatment; and (3) multiple focal-type AIP. In criterion (3), the multiple focal-type was defined by the presence of two or more focal lesions observed on DCE-CT or MRI, and lesions of AIP were separate (*i.e.* continuous lesion that involves at least one segment of the pancreas was previously considered to be a single lesion[14] and were thus excluded from this study).

From January 2001 to July 2020, 908 patients underwent pancreatic curative-intent surgery for PDA at our institution. To enable a 1:2 matching with the multifocal AIP group, we randomly chose 112 [78 men and 34 women; mean age ± SD, 61 ± 8.7 years (range: 38-86 years)] among the 613 patients with PDA who also underwent both DCE-CT and conventional MRI before surgery, using Statistic Package for Social Science software to generate the random numbers.

Imaging examinations

CT protocols: DCE-CT examinations were performed using 16-, 80-, or 320-detector CT scanner (Aquilion; Toshiba Medical Systems, Tokyo, Japan), using the following pancreatic scanning parameters: (1) detector collimation, 320.0 mm × 0.5 mm, 80.0 mm × 0.5 mm or 16.0 mm × 0.5 mm; (2) pitch, 1.0-1.4; (3) gantry rotation time, 0.35-0.50 s; (4) tube voltage, 120 kV; and (5) pancreatic reference tube current, 80-120 mA. All images were routinely reconstructed from the contrast-enhanced CT scans with thin-section images (slice thickness = 0.75-1.00 mm, reconstruction increment = 0.5 mm). Unenhanced and DCE-CT including the arterial, pancreatic parenchymal, and portal venous phases were acquired before and after intravenous administration of contrast agent. After unenhanced CT scanning, 300 mg/mL iodine (Ultravist 300; Schering; Bayer Healthcare Pharmaceuticals, Leverkusen, Germany) was injected intravenously using a power injector *via* an 18-gauge plastic antecubital catheter. An iodine dose of 1.5 mL/kg body weight was given at a flow rate of 4 mL/s. The administration of contrast agent was immediately followed by a flush of 30 mL saline. To accurately determine the timing for the standard triple-phase pancreatic imaging, a scanning

delay was performed based on an automated bolus-tracking technique offered by the supplier of the Toshiba CT system. Three phases enhanced scanning were obtained at a fixed delay of 20- to 25-s, 40- to 50- s, and 80- to 90-s after the contrast agent injection.

MR protocols: The MRI examinations were conducted using a 3.0 T imaging system (Magnetom Trio; Siemens Healthineers, Erlangen, Germany) with an 8-channel phased-array coil. The routine MR sequences for imaging the pancreas, including axial fat-suppressed T1-weighted turbo spin echo sequence, coronal T2-weighted (half-Fourier acquisition single-shot turbo spin echo) sequence, axial fat-suppressed T2-weighted turbo spin echo sequence, axial T1-weighted in-phase and out-of-phase sequences with fixed echo times of 1.4 and 2.8 ms, respectively, and DWI were performed at our institution. Axial single-shot echo-planar DWI sequence was completed using the following scanning parameters: repetition time, 6900 ms; echo time, 73 ms; intersection gap, 6 mm; slice thickness, 3 mm; field of view, 350 mm × 350 mm; matrix size, 128 mm × 128 mm; flip angle, 90°; partial Fourier factor, 6/8; parallel imaging reduction factor, 2; bandwidth, 2441 Hz per pixel; and the number of excitations, 8 (water excitation technique with b-value of 50 s/mm^2 and 800 s/mm^2). Axial fat-suppressed T1WI was performed with the following parameters: echo time, 1.4 ms; repetition time, 3.9 ms; flip angle 9°; slice thickness, 3 mm; matrix, 320 mm × 182 mm; field of view, 250 mm × 280 mm; and the number of excitations, 1. DWI and axial fat-suppressed T1WI sequences were performed with breath hold and free breathing, respectively. The main scan parameters of the MR sequences were presented in our earlier paper[13]. Apparent diffusion coefficient values (ADC) maps were automatically generated from DW images, and ADC values were calculated on a Syngo workstation (Syngo Multimodality Workplace; Siemens Healthineers). One radiologist, who had precise knowledge of the lesion's location based on the overall findings from the radiological images and pathological and surgical datasets, retrospectively manually drew and placed the largest possible regions of interest within the pancreatic lesion and surrounding parenchyma. ADC values of pancreatic lesions were measured and compared with the surrounding parenchyma.

Image analysis: The detection of each multifocal AIP lesion was divided into three image sets: axial fat-suppressed T1WI, DWI, and DCE-CT. Each image set was independently analyzed on a picture archiving and communication workstation (Shida picture archiving and communication system, Fuzhou, China) with the free adjustment of window width and level by two abdominal radiologists (21 and 7 years of experience in interpreting abdominal CT/MRI). The two radiologists knew that this study was being conducted to detect the lesions of multifocal AIP but were unaware of any clinical information, biological data, other conventional imaging findings, histopathological, and surgical results. The radiologists identified the absence or presence of the lesion with a 5point diagnostic confidence scales as follows: Score 1, definitely absent; Score 2, probably absent; score 3, indeterminate; Score 4, probably present; and Score 5, definitely present. In cases with diagnostic confidence score of 4 or 5, a lesion was present, and the location of the lesion was recorded on a report sheet. The signal intensity of the lesion was assessed on axial fat-suppressed T1WI and DWI image sets compared with the surrounding parenchyma and was categorized as low, intermediate, or high.

One month after the completion of the detection rating for all patients of multifocal AIP, two radiologists subsequently analyzed the DCE-CT and MRI images of all 168 patients (56 AIP and 112 PDA) in a random sequence and determined, in consensus, the absence or presence of the following characteristic imaging features as previous reports[4,16-18] for differentiating between AIP and PDA: multiple pancreatic masses; ill-defined margin; delayed homogeneous enhancement; capsule-like rim; marked upstream MPD dilation; upstream pancreatic atrophy; multiple MPD strictures; and extrapancreatic manifestations, including vascular invasion, peripancreatic fat infiltration, and peripancreatic and retroperitoneal lymphadenopathy.

Laboratory data: The serum immunoglobulin G fraction 4 (IgG4) concentrations, if available, were carefully recorded.

Statistical analysis

Statistical analysis was performed using Statistic Package for Social Science (version 19.0; IBM Corp., Armonk, NY, United States) software. Quantitative variables were summarized as the mean ± SD. Descriptive variables were determined using percentages and medians. The Wilcoxon signed rank test was performed to analyze the mean ADC values in multifocal AIP lesions and the surrounding parenchyma and to compare the ADC values in multifocal AIP and PDA. Prior to determining the

agreement between the two radiologists with respect to confidence ratings for a detection task of multifocal AIP lesions, κ-values were used to assess interobserver agreement interpreted as follows: 0.81-1.00, excellent agreement; 0.61-0.80, good agreement; 0.41-0.60, moderate agreement; and 0.21-0.40, fair agreement[19,20]. Friedman's nonparametric test was performed to evaluate the differences of diagnostic confidence level among axial fat-suppressed T1WI, DWI, and DCE-CT image sets for each radiologist. The frequency of the characteristic imaging findings for differentiating multifocal AIP from PDA was compared *via* the chi-squared or McNemar's test, as appropriate. A two-tailed P value of < 0.05 was deemed to be a statistically significant difference.

RESULTS

Overall profile of 26 patients with multifocal AIP at our center

Clinical findings: From 26 patients, 18 were female, and 8 were male. The median age was 60.7 ± 17.0 years (range: 26-89 years). Among 118 patients with AIP, 54 (45.8%) showed diffuse-type AIP, 38 (32.2%) showed unifocal-type AIP, and twenty-six cases (22.0%) showed multifocal-type AIP. Of the 26 patients with multifocal-type AIP, 4 (15.3%) were associated with Crohn's disease, and 2 (7.7%) were associated with ulcerative colitis (Figure 1). A total 56 lesions were found in these 26 patients with multifocal AIP. Twenty-two patients had two lesions, and four patients had three lesions. The lesions of 16 patients were in the pancreatic head/uncinate process and tail. The lesions of 6 patients were in the pancreatic head and body; 4 patients with 3 lesions each were in the pancreatic head, body, and tail. Pancreatic tissue samples were obtained in 18 patients (69.2%) including three from surgical resection, five from endoscopic ultrasound-guided fine-needle aspiration biopsy, and ten from CT guided percutaneous biopsy. All patients were affected by type 1 AIP.

The serum concentrations of IgG4 (0.03-2.01 g/L) were elevated above the normal limit in 94.9% (112/118) of the patients with AIP, 96.2% (25/26) of the patients with multifocal-type AIP, 96.3% (52/54) of the patients with diffuse-type AIP, and 92.1% (35/38) of the patients with unifocal-type AIP. The mean serum concentration of IgG4 of the patients with multifocal-type AIP was 14.77 ± 8.05 g/L. IgG4 levels were not elevated in 53 patients with PDA, and the remaining 59 patients with PDA had no measurement of serum IgG4 before surgery.

Treatment and relapse of patients with multifocal-type AIP: Initial treatment of all patients with multifocal AIP consisted of steroid therapy in 23 patients (88.5%) or surgical resection in three patients (11.5%). After the diagnosis was rendered, 22 patients received treatment with high-dose steroid. They received an intravenous injection of daily methylprednisolone 40-80 mg for 3 to 7 d, after which 10-20 mg of oral prednisone was given per day. During the median 8.6 mo (interquartile range: 5.1 to 23.0 mo) of follow-up, all patients who underwent steroid treatment with/without surgical resection showed complete radiological and clinical remission. The remaining 4 patients refused steroid treatment and were lost to follow-up.

Diagnostic performances of DWI, axial fat-suppressed T1WI, and DCE-CT

The senior and junior radiologist detected 50/56 and 46/56 lesions on DWI, 34/56 and 38/56 on axial fat-suppressed T1WI, and 22/56 and 24/56 on DCE-CT, respectively. DWI MRI had the highest relative sensitivity [4650/56 (82.1%89.3%) lesions] in the detection of lesions in multifocal AIP (Figures 2 and 3).

Statistically significant differences of the confidence level score were observed among these three image sets for the two radiologists. Diagnostic confidence level score of DWI alone was the highest, while that of DCE-CT was the lowest for each radiologist (Table 1). Weighted κvalues that rated the confidence levels of the readers in terms of image interpretation are summarized in Table 2. Good interobserver agreement was observed between the two radiologists for determining the presence or absence of lesions on DWI datasets (weighted κ = 0.878). Moderate interobserver agreement was observed between two readers on axial fat-suppressed T1WI and DCE-CT image datasets (weighted κ = 0.742 and weighted κ = 0.683, respectively).

The mean ADC values ($\times 10^{-3}$ mm^2/s) of multifocal AIP lesions and the surrounding pancreatic parenchyma were 0.96 ± 0.14 (range: 0.681.26) and 1.15 ± 0.15 (range: 0.891.40), respectively. ADC values of the AIP lesions were significantly lower compared with those of the surrounding pancreatic parenchyma according to the Wilcoxon signedrank test (P = 0.001; Table 3).

Table 1 Friedman analysis of variance by ranks for the diagnosis confidence level score

Groups	Junior radiologist	Senior radiologist
DWI	2.32	2.32
Axial-suppressed T1WI	1.95	2.07
Dynamic contrast enhanced CT	1.73	1.61
P value	0.033	0.004

CT: Computed tomography; DWI: Diffusionweighted imaging; T1WI: T1 weighted image.

Table 2 Interobserver agreement in image interpretation

Groups	*κ*-value
DWI	0.878
Axial fat-suppressed T1WI	0.742
Dynamic contrast enhanced CT	0.683

CT: Computed tomography; DWI: Diffusionweighted imaging; T1WI: T1 weighted image.

Table 3 Apparent diffusion coefficient values of autoimmune pancreatitis lesions compared with those of surrounding pancreatic tissue[1]

	AIP lesions ADC	Surrounding parenchyma ADC	Wilcoxon signed-rank test	
	Values (n = 56) ($\times 10^{-3}$ mm^2/s)	Values (n = 56) ($\times 10^{-3}$ mm^2/s)	Z value	P value
Mean	0.96	1.15	-5.11	0.001
SD	0.14	0.15		
Median	0.96	1.16		
Minimum	0.68	0.89		
Maximum	1.26	1.40		

[1]Apparent diffusion coefficient values in autoimmune pancreatitis lesions were lower (0.96 ± 0.14) compared with those in the surrounding pancreatic parenchyma (1.15 ± 0.15), Wilcoxon signed-rank test demonstrated this difference to be statistically significant (P = 0.001). ADC: Apparent diffusion coefficient; AIP: Autoimmune pancreatitis; SD: Standard deviation.

Evaluation of radiological findings between multifocal-type AIP and PDA

Fifty-six patients with multifocal-type AIP and 112 patients with PDA were compared. The differences in the imaging features between multifocal-type AIP and PDA are summarized in Table 4. Multifocality (100% *vs* 1.7%, $P < 0.01$), capsule-like rim (21.4% *vs* 3.6%, $P < 0.01$), multiple MPD strictures (47.3% *vs* 1.8%, $P < 0.01$), and delayed homogeneous enhancement (92.8% *vs* 13.4%, $P < 0.01$) were significantly more frequent in multifocal AIP. Peripancreatic fat infiltration (46.4% *vs* 91.0%, $P < 0.01$), vascular invasion (14.3% *vs* 71.4%, $P < 0.01$), upstream pancreatic atrophy (19.6% *vs* 58.9%, $P < 0.01$), peripancreatic and retroperitoneal lymphadenopathy (3.6% *vs* 61.6%, $P < 0.01$), and marked upstream MPD dilatation (3.6% *vs* 52.7%, $P < 0.01$) were significantly more frequent in PDA than in multifocal AIP. ADC values of the multifocal AIP lesions were significantly lower than that of PDA. However, the indistinct margin between multifocal AIP and PDA was not statistically different. Furthermore, the capsule-like rim was present with a lower frequency (21.4%) in multifocal AIP in our study. Additionally, three multifocal AIP patients underwent ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography/CT, and increased FDG uptake was noted in seven lesions in the whole pancreas (Figures 2 and 3).

Table 4 Comparison of radiological findings between patients with multifocal autoimmune pancreatitis and pancreatic ductal adenocarcinoma, *n* (%)

	Multifocal AIP	PDA	*P* value
No. of radiological findings of the pancreas	56	112	
Multifocal lesion	56 (100)	2 (1.7)	< 0.01
Capsule-like rim	12 (21.4)	4 (3.6)	< 0.01
Delayed homogeneous enhancement on MDCT	52 (92.8)	15 (13.4)	< 0.01
Margin (indistinct)	48 (85.7)	89 (79.5)	0.325
Peripancreatic fat infiltration	26 (46.4)	102 (91.0)	< 0.01
Vascular invasion	8 (14.3)	80 (71.4)	< 0.01
Multiple MPD strictures	53 (47.3)	2 (1.8)	< 0.01
Marked upstream MPD dilatation	2 (3.6)	59 (52.7)	< 0.01
Upstream pancreatic atrophy	11 (19.6)	66 (58.9)	< 0.01
Lymphadenopathy	2 (3.6)	69 (61.6)	< 0.01
ADC values ($\times 10^{-3}$ mm^2/s)	0.96 ± 0.16	1.15 ± 0.14	< 0.01

ADC: Apparent diffusion coefficient; AIP: Autoimmune pancreatitis; MDCT: Multidetector computed tomography; MPD: Main pancreatic duct; MRI: Magnetic resonance image; PDA: Pancreatic ductal adenocarcinoma.

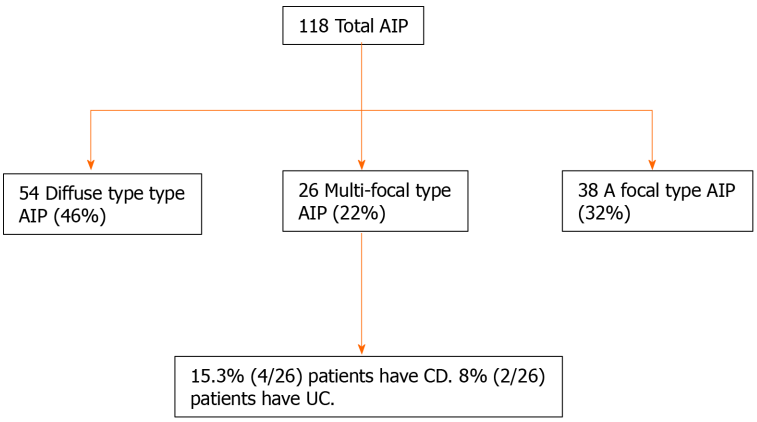


Figure 1 Flowchart of patients with multifocal autoimmune pancreatitis have Crohn's disease and ulcerative colitis. AIP: Autoimmune pancreatitis; CD: Crohn's disease; UC: Ulcerative colitis.

DISCUSSION

AIP is an uncommon form of chronic pancreatitis that typically affects men in their fifth to sixth decades and usually presents with pancreatic enlargement and obstructive jaundice, with a reported incidence rate of up to 6% in chronic pancreatitis patients[1,21,22]. The most common radiological manifestation of AIP is a grossly enlarged sausage-shaped hypoattenuating pancreas with homogeneous decreased enhancement at the arterial phase and delayed rim-like enhancement at the portal venous phase. The unifocal form of AIP at the pancreatic head is by far the second-most common type[6]. As in our cases, the most uncommon type is the multifocal form that has only been described in a few reports[3,10,23]. All patients with multifocal AIP in our study were type 1 and responded well to steroid therapy, consistent with previous study results.

In this cohort of 118 patients of AIP, 22.0% showed multifocal-type AIP, suggesting it is not as uncommon as previously thought in actual clinical practice. One possible reason for overlooking is the radiologists have been extremely unfamiliar with its characteristic imaging features. Another possible reason may be attributed to some CT and MR imaging findings of unifocal-type or diffuse-type AIP may overlap with multifocal-type AIP. It has also been hypothesized that different diagnostic

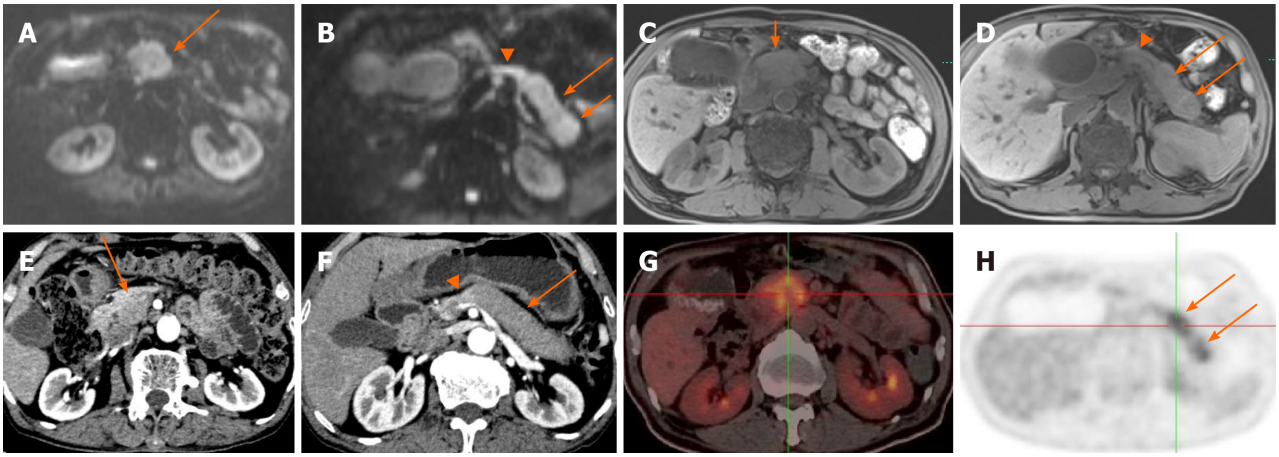


Figure 2 A 72-year-old man with multifocal autoimmune pancreatitis. A-D: Diffusionweighted imaging and axial fat-suppressed T1 weighted image showed three indistinct mass-like swelling (arrows) in the pancreatic head, body, and tail; E: The pancreatic head lesion (arrow) could not be clearly shown on dynamic contrast enhanced-computed tomography; F: Dynamic contrast enhanced-computed tomography could only show a merged lesion in the pancreatic body/tail (arrow); G-H: Positron emission tomography-computed tomography images showed three focal intense uptakes of fluorodeoxyglucose in the whole pancreas, which were not detectable on dynamic contrast enhanced-computed tomography. B, D, and F: The computed tomography and magnetic resonance images demonstrated relatively normal volume of pancreas (arrowheads) in the pancreatic neck.

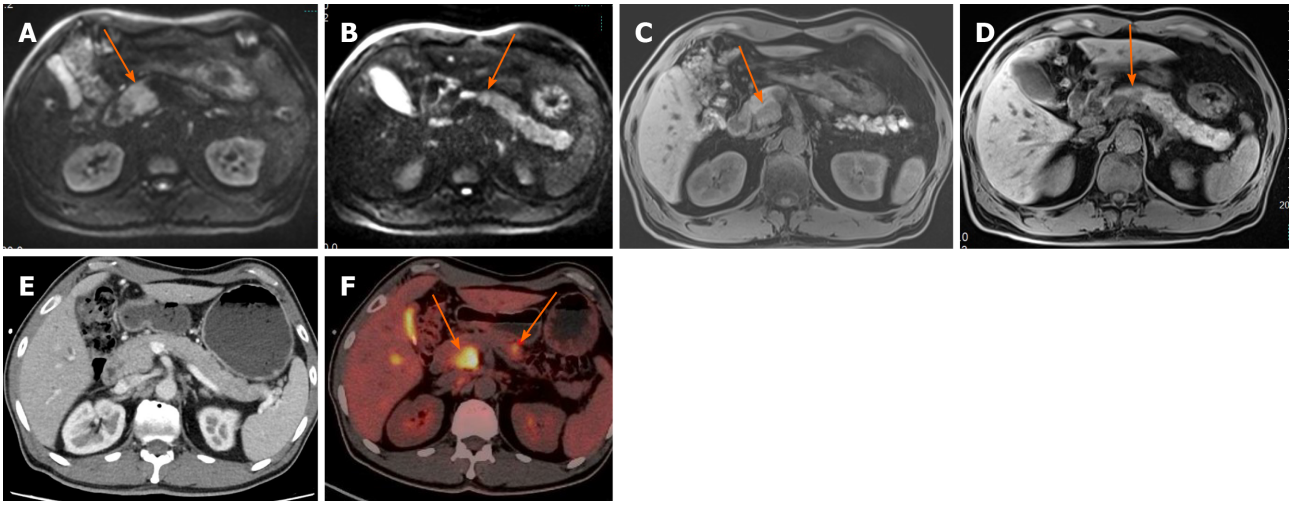


Figure 3 A 68-year-old man with multifocal autoimmune pancreatitis. A-D: Diffusionweighted imaging and axial fat-suppressed T1 weighted image showed two indistinct mass-like swelling (arrows) in the pancreatic head and body; E and F: Positron emission tomography-computed tomography image showed two focal intense uptakes of fluorodeoxyglucose in the pancreatic head and body, which were not detectable on dynamic contrast enhanced-computed tomography.

approaches in China may greatly contribute to the relatively lower incidence rate of multifocal AIP.

Current diagnostic methods available for the differentiation of pancreatic lesions are various, among which contrast-enhanced ultrasonography, endoscopic ultrasonography (EUS), contrast enhanced-CT, contrast enhanced-MRI, contrast-enhanced elastography, EUS-fine-needle aspiration, and contrast enhanced-EUS have been widely used in recent years. As one of the current gold standards for diagnosis of pancreatic neoplasms, EUS- fine-needle aspiration with 100% specificity can correctly access the pathological diagnosis directly. In addition, an accurate tissue diagnosis based on a EUS- fine-needle aspiration biopsy specimen may render surgery for those benign neoplasms or indolent cancer unnecessary. However, the best method for pancreatic mass diagnosis remains unclear[24]. In our country, DCE-CT, diagnostic endoscopic retrograde pancreatography, endoscopic biopsy, and the following therapeutic trial of steroids have been more actively conducted in the management of patients with suspected AIP than before[9,14,25]. Multifocal AIP can be mistakenly classified as diffuse or unifocal AIP when only using CT, as our study results showed that DWI and axial fat-suppressed T1WI have higher sensitivity than DCE-CT to find AIP lesions.

A literature search with the medical subject headings term 'autoimmune pancreatitis' in PubMed, Wanfang Database, and the China National Knowledge Infrastructure was performed by authors (Zhen-Shan Shi) on August 8, 2020. The reference lists of relevant studies were searched to identify other additional eligible articles. A PubMed search of the past 20 years revealed 783 articles describing focal-type AIP in the English and Chinese literature. However, most reported studies of focal AIP have focused on the imaging features of unifocal and not multifocal; only 18 articles involved the imaging features of multifocal-type AIP. Most of these studies were small case series or single case reports. A total of 132 cases of multifocal AIP, including our current cases, were retrospectively analyzed and summarized in the present study. We found that multifocal AIP accounted for 22% of total AIP, and most patients presented above the age of 60 years with more females than males[1,21]. The gender difference of multifocal AIP was not consistent with that of total AIP with more males than females.

In our study, 23.3% of multifocal AIP patients had associated IBDs (15.3% had Crohn's disease, and 7.7% had ulcerative colitis) and were type 1 AIP. Most cases with coexisting AIP and IBD were type 2 AIP in the previous studies[2,26]. The possible reason is type 1 AIP is significantly more frequent in East Asia, and type 2 AIP generally prevails in Western countries[26]. Our data suggest that elevated serum IgG4 concentrations are also helpful in identifying multifocal AIP, like in previous studies[4,27].

This study confirmed the high sensitivity (82%89%) of DWI for the detection of AIP lesions[7,28], which also provided an important contribution to the highest radiologist diagnostic confidence score and the highest level of interobserver agreement (weighted κ = 0.878) between the two radiologists and also demonstrated that DWI was the optimal image sequence among the three image sets (DWI, axial fat-suppressed T1WI, and DCE-CT). We speculate that the lowest confidence score and interobserver agreement of DCE-CT between the two radiologists with different experience may be due to the inferior resolution in showing the slight difference of CT value between AIP lesions and the surrounding pancreas, which may make images more difficult to interpret than with DWI and axial fat-suppressed T1WI. Conversely, MRI has the excellent soft tissue contrast resolution, and DWI has outstanding imaging capability to detect pancreatic focal lesions, even minor lesions[29,30].

A significant difference in ADC value was also found between multifocal AIP lesions and the surrounding pancreas, similar to the results of unifocal-type AIP[4]. We hypothesize that DWI, which can be conducted without contrast media and in a relatively short time, will become a crucial imaging technique in the detection of AIP lesions.

Our present study showed that seven key distinguishing features of PDA and multifocal AIP were as follows among various CT and MR findings, particularly for multifocal AIP: (1) multiple pancreatic lesions; (2) hypoattenuating capsule-like rim; (3) delayed homogeneous good enhancement; (4) absence of distal pancreatic atrophy; (5) mild upstream MPD dilatation; (6) multiple MPD strictures; and (7) a lower ADC value are more frequently observed in multifocal AIP like other prior reports of unifocal-type AIP, which could be deemed reliable findings to prevent unnecessary invasive procedures and surgical treatment in the management of the suspected patients.

On the contrary, peripancreatic fat infiltration, vascular invasion, and peripancreatic and retroperitoneal lymphadenopathy are more often observed in PDA. Additionally, even with ^{18}F-FDG-positron emission tomography/CT examination, when the pancreatic lesion is accurately localized, it can be often confused with PDA because it has been recently reported to show increased FDG uptake at the locally affected lesions of AIP[31-33], which is also an observation from our study. We consider that the use of these seven key differentiating features can be helpful in supporting the diagnosis of AIP when the clinical, laboratory, and other imaging features are equivocal for distinguishing between focal-type AIP and PDA.

We found that there was no statistically significant difference between multifocal AIP and PDA with respect to ill-defined margin, as in previous studies[4,16]. The capsule-like rim or halo sign has already been reported as a specific feature of AIP, with a varying frequency up to 63%[5,14,34]. This sign was observed with lower frequency (21%) in multifocal AIP in our study. We presume that multifocal-type AIP is the early manifestation of AIP, and the multiple lesions will be merged into diffuse-type along with the progression of this entity. Then, typical or uncommon imaging features will be typical, and the halo sign may be more frequently seen. However, further work is needed to confirm this hypothesis.

Our study has a few limitations. First, this study was performed by a retrospective research design, and the number of cases is relatively small. However, AIP itself is a rare entity, and multifocal AIP is even rarer. Second, the incidence rate of multifocal AIP in this study was at a single tertiary center with a specialized care clinic that has been widely recognized for its expertise and experience with pancreatic diseases in our country and may confer a considerable potential referral bias with an overestimated frequency of multifocal AIP. Third, the blinded radiologists had initially determined patient diagnosis in daily clinical practice; recall bias might have occurred. Last, we used a variety of CT scanners with different detectors (*e.g.*, 320-, 80- and 16- detectors) from the same manufacturer with scan parameters that slightly varied from each other, which may affect the diagnostic performance among these modalities.

CONCLUSION

In conclusion, multifocal AIP is not as rare as previously thought. DWI as part of pancreatic conventional MRI protocol has the potential benefit in the detection of AIP lesions. The combinations of CT and MRI imaging features may be helpful for differentiating multifocal AIP from PDA by the key distinguishing findings, including multiple pancreatic lesions, capsule-like rim, delayed homogeneous enhancement, multiple MPD strictures, and lower ADC values.

ARTICLE HIGHLIGHTS

Research background

Multifocal-type autoimmune pancreatitis (AIP), sometimes forming multiple pancreatic masses, is frequently misdiagnosed as pancreatic malignancy in routine clinical practice. It is critical to know the imaging features of multifocal-type AIP to prevent misdiagnosis and unnecessary surgery. To the best of our knowledge, there have been no studies evaluating the value of diffusionweighted imaging (DWI), axial fat-suppressed T1 weighted image (T1WI), and dynamic contrast enhanced-computed tomography (DCE-CT) in detecting the lesions of multifocal-type AIP.

Research motivation

The key issue is whether CT and magnetic resonance imaging features of multifocal-type AIP can help definitively distinguish from pancreatic ductal adenocarcinoma (PDA) and whether there is an optimal modality to identify pancreatic lesions as correctly as possible. The results will provide important information on the diagnostic performances of DWI, axial fat-suppressed T1WI, and DCE-CT and the key imaging features for differentiating multifocal-type AIP from PDA.

Research objectives

We aimed to clarify the exact prevalence and radiological findings of multifocal AIP in our cohorts and compare the sensitivity of DWI, axial fat-suppressed T1WI, and DCE-CT for detecting AIP lesions. We also compared radiological features between multifocal AIP and PDA with several key imaging landmarks.

Research methods

Twenty-six patients with proven multifocal AIP were retrospectively included. Two blinded independent radiologists rated their confidence level in detecting the lesions on a 5-point scale and assessed the diagnostic performance of DWI, axial fat-suppressed T1WI, and DCE-CT. CT and magnetic resonance images of multifocal AIP were systematically reviewed for typical imaging findings and compared with the key imaging features of PDA.

Research results

Among 118 patients with AIP, 26 (22.0%) had multiple lesions (56 lesions). Ulcerative colitis was associated with multifocal AIP in 7.7% (2/26) of patients, and Crohn's disease was associated in 15.3% (4/26) of patients. In multifocal AIP, multiple lesions, delayed homogeneous enhancement, multifocal strictures of main pancreatic duct, capsule-like rim, lower apparent diffusion coefficient values, and elevated serum Ig4 Level were observed significantly more frequently than in PDA, whereas the presence

of capsule-like rim in multifocal-type AIP was lower in frequency than total AIP. Of these lesions of multifocal AIP, DWI detected 89.3% (50/56) and 82.1% (46/56) by the senior and junior radiologist, respectively.

Research conclusions

Multifocal AIP is not as rare as previously thought and was seen in 22% of our patients. The diagnostic performance of DWI for detecting multifocal AIP was best followed by axial fat-suppressed T1WI and DCE-CT.

Research perspectives

Larger and longer term prospective studies to investigate the important radiological findings for differential diagnosis between multifocal AIP and PDA should be performed in future studies.

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ORIGINAL ARTICLE

Retrospective Study

Recent trends in the prevalence and distribution of colonic diverticula in Japan evaluated using computed tomography colonography

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Abstract

BACKGROUND

Computed tomography colonography (CTC) may be superior to colonoscopy and barium enema for detecting diverticula. However, few studies have used CTC to diagnose diverticula.

AIM

To evaluate the current prevalence and distribution of colonic diverticula in Japan using CTC.

METHODS

This study was conducted as part of the Japanese National Computed Tomographic Colonography Trial, which included 1181 participants from 14 hospitals in Japan. We analyzed the prevalence and distribution of colonic diverticula and their relationships with age and sex. The relationship between the diverticula and the length of the large intestine was also analyzed.

RESULTS

Diverticulosis was present in 48.1% of the participants. The prevalence of diverticulosis was higher in the older participants ($P < 0.001$ for trend). The diverticula seen in younger participants were predominantly located in the right-sided colon. Older participants had a higher frequency of bilateral type (located in the right- and left-sided colon) diverticulosis ($P < 0.001$ for trend). The length of the large intestine with multiple diverticula in the sigmoid colon was significantly shorter in those without diverticula ($P < 0.001$).

CONCLUSION

The prevalence of colonic diverticulosis in Japan is higher than that previously reported. The prevalence was higher, and the distribution tended to be bilateral in older participants.

Key Words: Computed tomography colonography; Diverticulosis; Diverticular disease; Colon

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Core Tip: In this retrospective study, we evaluated the current prevalence and distribution of colonic diverticula in Japan using computed tomography colonography. Diverticulosis was present in 48.1% of the 1181 participants. The prevalence of diverticulosis was higher among the older participants. The diverticula seen in younger participants were predominantly located in the right-sided colon. Older participants had a higher frequency of the bilateral type. The length of the large intestine with multiple diverticula in the sigmoid colon was significantly shorter in participants without diverticula.

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INTRODUCTION

Colonic diverticulosis is a common abnormality of the colon. Almost all colonic diverticula are pseudodiverticula, which are small outpouchings of the colonic mucosa and submucosa that herniate through the muscle layers at sites of vascular perforation [1]. Diverticulosis refers to asymptomatic diverticula, whereas diverticular disease

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refers to symptomatic diverticula. Diverticular disease is classified into uncomplicated and complicated diseases, including perforation, fistulae, obstructions, and bleeding [1]. The cumulative incidences of bleeding and diverticulitis in patients with colonic diverticulosis are 10% and 10%-25%, respectively[2,3]. Additionally, the prevalence of colonic diverticulosis increases with age[1,4,5]. Due to Japan's aging population, the incidence of diverticular diseases in older patients who need treatment will also likely increase[6].

Findings from studies involving colonoscopy or barium enema have shown that the prevalence and distribution of diverticulosis differ according to country, region, and race[7-11]. These reports are from the early 2000s until the mid-2010s, and also reported that the incidence of diverticula increased with time[5,8]. Colonic diverticula occur mainly in the left colon in Western populations and in the right colon in Japanese and other Asian populations[7-9,11,12]. Recent studies have reported that the overall prevalence of diverticulosis in Japan is 20%-26.0%[5,7,8].

Colonoscopy is a standard technique that is widely performed for the screening and diagnosis of colonic and rectal diseases. However, there are reports suggesting that compared to colonoscopy, assessments based on barium enema are superior for diagnosing colonic diverticula, especially in the sigmoid colon[13,14]. In recent years, computed tomography colonography (CTC) has been widely used as an alternative colon cancer screening method. CTC can detect diverticula as outpouchings of the colonic wall from a more advantageous perspective, and it may be superior to colonoscopy and barium enema for detecting diverticula. Only one large study has evaluated the prevalence and distribution of colonic diverticula using CTC[15]. Hence, this study is the largest study in the world and is the first to accurately evaluate the prevalence and distribution of colonic diverticula in Japanese patients using CTC.

MATERIALS AND METHODS

This study was conducted as a part of another study on the application of CTC for detecting polypoid lesions [Japanese National Computed Tomographic Colonography Trial (JANCT)][16]. The trial was registered with Clinical Trials. gov (No. NCT00997802) and the UMIN Clinical Trials Registry (No. UMIN000002097). The medical ethics committee of Fukushima Medical University reviewed and approved the study design. The JANCT data were analyzed using the Digital Imaging and Communications in Medicine protocol. Patients from 14 hospitals in Japan were enrolled between September 2009 and August 2011. This study was conducted in accordance with the principles of the Declaration of Helsinki. The institutional review board of each hospital approved the study. All patients provided written informed consent prior to enrollment. The patients included in this study underwent CTC for screening purposes or because they had abdominal symptoms, positive fecal immunochemical test (FIT) results, a personal history of polyps, or a familial history of colorectal cancer (CRC) or polyps. Moreover, 847 participants were screened, 96 for abdominal symptoms or change in bowel habits, 165 for positive FIT, 42 for melena or hematochezia, 11 for surveillance after polypectomy, and 19 for abnormal blood test results (anemia or tumor marker elevation). The exclusion criteria have been described in detail previously[16]. Briefly, participants with serious medical conditions for bowel preparation and colonoscopy or CTC, who underwent colonoscopy or barium enema during the preceding 3 years, with known colorectal polyps or cancers, a history of inflammatory bowel disease, and familial polyposis, and who underwent colorectal surgery were excluded from the study.

Computed tomography colonography

Details of the CTC procedure have been previously published[16]. A polyethylene glycol electrolyte lavage solution (Niflec; Ajinomoto Pharmaceuticals, Tokyo, Japan) was used for bowel preparation, and contrast medium containing sodium diatrizoate (Gastrografin; Bayer Yakuhin, Osaka, Japan) was added to tag the residual fluid. A tube was inserted while the patients were in the left decubitus position before CO_2 insufflation and CTC scanning. CO_2 insufflation was performed using an automated CO_2 insufflator (HP-2; Horii Pharmaceutical Industries, Ltd., Osaka, Japan). All CTC examinations were performed using 64- or 16-channel multidetector row computed tomography (CT) scanners. CT scanning was performed under single breath-holds while the patients were in the supine and prone positions.

Evaluation of the diverticula using computed tomographic colonography

Four radiologists and six gastroenterologists participated in this study as readers of the CTC images. The readers analyzed CTC images from a median of 1750 patients (range: 400–8000 patients). Identification of the colonic diverticula and measuring the length of the large intestine were performed at workstations at each hospital, namely: AZE Virtual Place (AZE, Tokyo, Japan), Synapse Vincent (Fujifilm Medical Co., Tokyo, Japan), and Ziostation (Ziosoft, Tokyo, Japan). Axial 2-dimensional (2D) images, captured while the patients were in the supine position, were primarily used to determine the number of diverticula. A diverticulum was identified as an outpouching from the colonic wall. If necessary, other 2D and 3-dimensional (3D) images, comprising luminal fly-through or air-contrast barium enema images, were analyzed (Figure 1). The readers recorded the number of diverticula in each colonic segment, including the cecum and as well as the ascending, transverse, descending, sigmoid, and rectosigmoid colons. The rectosigmoid colon was defined as the segment between the promontrium and the inferior margin of the second sacral vertebra. Diverticula in the terminal ileum and appendix were also evaluated. The terminal ileum was defined as the portion of the ileum 10 cm from the ileocecal valve. If there were < 20 diverticula, the readers recorded the numbers as is; if there were > 20 diverticula, the readers recorded their findings as severe. When diverticula were found in multiple colonic segments, we evaluated the number of diverticula in each segment in duplicate. In analysis, the grading of diverticula was in accordance with a previous study to allow for comparison with the previous study[15]. The number of diverticula was graded in each segment: 1-5 diverticula were classified as rare, 6-20 diverticula were classified as multiple, and more than 20 diverticula were classified as severe. The length of the large intestine was defined as the distance from the anus to the appendiceal orifice and was measured automatically using 3D workstations.

Endpoints

The primary endpoint of this study was to clarify the prevalence of colonic diverticulosis by CTC and to evaluate it by sex, age group, and segment. There were two secondary endpoints; the first of which was to investigate the prevalence of diverticulosis in the terminal ileum and appendix (which have not been evaluated accurately so far), and the second was to investigate the relationship between diverticulosis and the length of the large intestine.

Statistical analyses

Continuous variables are reported as medians and ranges. To evaluate differences in the prevalence of diverticula and the length of the large intestine, the Mann-Whitney *U* test for unpaired samples was performed. We used the chi-squared test to assess the relationship between the prevalence of diverticulosis and sex. Trends in age-related difference and increases in the prevalence of diverticula were evaluated using the Cochran-Armitage test. Statistical significance was set at $P < 0.05$. Test of normal distribution was evaluated using Shapiro-Wilk test. All statistical analyses were performed using IBM® SPSS® software, version 26 (IBM Corp., Armonk, NY, United States), except for the Cochran-Armitage test and Shapiro-Wilk test, which was performed using Bell Curve for Excel version 2.20 (Social Survey Research Information Co., Ltd., Tokyo, Japan).

RESULTS

Characteristics of the participants and relationship between age, sex and location

Our study population comprised 1181 participants, including 669 men (56.6%) and 512 women (43.4%) with a median age of 62 years (range: 23-91 years). Of the 1181 participants, 568 (48.1%) had ≥ 1 diverticula. The median age of the participants with diverticula was 64 years (range: 27-91 years); that of participants with no diverticulum was 60 years (range: 23-84 years). Participants with diverticula were significantly older than participants with no diverticulum ($P < 0.001$) (Table 1). The participants were stratified into five groups according to age. There were 108 participants in the 40 years or younger group, 165 in the 41-50 years group, 268 in the 51-60 years group, 327 in the 61-70 years group, and 313 in the 71 years and over group. A normal distribution of age dates was performed.

Diverticulosis was present in 346 men (29.3%) and 222 women (18.8%). The frequency of diverticulosis in men was significantly higher than that in women (P =

Table 1 Characteristics of the participants

	Diverticula positive (*n* = 568)	Diverticulum negative (*n* = 613)	*P* value
Age: Median (range)	64 (27-91)	60 (23-84)	< 0.001
Sex, *n* (%): Male:female	346 (29.3):222 (18.8)	323 (27.3):290 (24.6)	0.004
Age group, *n* (%): Male:female			
≤ 40 (*n* = 108)	27 (25.0):1 (0.9)	56 (51.9):24 (22.2)	0.004
41-50 (*n* = 165)	42 (25.5):19 (11.5)	56 (33.9):48 (29.1)	0.058
51-60 (*n* = 268)	80 (29.9):50 (18.7)	67 (25.0):71 (26.5)	0.033
61-70 (*n* = 327)	97 (29.7):80 (24.5)	88 (26.9):62 (19.0)	0.485
≥ 71 (*n* = 313)	100 (31.9):72 (23.0)	56 (17.9):85 (27.2)	0.001

Characteristics of the 1181 participants analyzed in this study. The prevalence of diverticulosis according to age group and the relationship between diverticulosis and sex were analyzed.

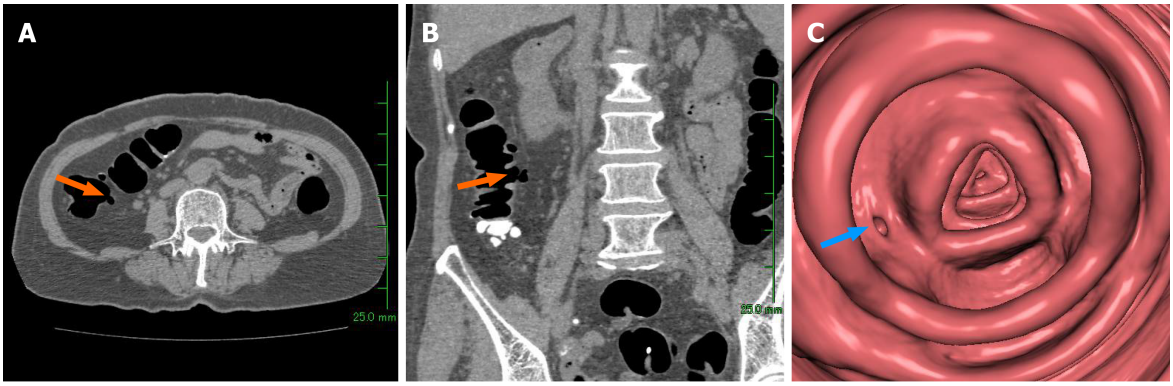


Figure 1 Detection of diverticula using computed tomography colonography. The diverticula were detected mainly in 2-dimensional (2D) supine axial images. If necessary, other 2D or 3-dimensional (3D) images were used. The arrow indicates a diverticulum. A: 2D-supine axial image; B: 2D-supine coronal image; C: 3D-virtual colonoscopy image.

0.004). The frequency of diverticulosis was significantly higher or tended to occur in men in other age groups, but did not differ between sexes in the 60s. The distribution of diverticula and the relationship between sex and location of diverticula are also shown in Table 2. Diverticula were most often found in the ascending colon. The frequency of diverticulosis in men was higher in all segments except the rectosigmoid colon. Regarding the relationship between age and frequency of diverticulosis, the prevalence of diverticulosis significantly increased with age ($P < 0.001$ for trend) (Figure 2). Over half of the participants aged > 60 years had diverticula.

Distribution and grade of diverticula in each age group

The prevalence and the number of diverticula in each colonic segment increased with age, and diverticula were also found in the terminal ileum and appendix (Table 3). Thirteen participants (1.1%) had diverticula in the terminal ileum, and all had less than six diverticula. The appendix was visualized in 680 participants (57.6%), six (0.5%) of whom had appendiceal diverticula. Five participants had one diverticulum, with one participant in his 60s having three diverticula in the appendix.

Relationship between age and location of diverticula

Participants with diverticula were divided into three groups according to the location of the diverticula: The right-sided type was defined as diverticula located from the ileum to the transverse colon only; the left-sided type was defined only from the descending to rectosigmoid colon; and the bilateral type was defined as located on both sides of the colon. We investigated the proportion of diverticula in each type in all patients (Figure 3). As mentioned above, the prevalence of diverticula increased with age, while the prevalence of right-sided and left-sided diverticula did not differ

Table 2 Relationship between sex and location of diverticia

Segment	*n* (%)	Male:Female
Ileum	13 (1.1)	9:4
Appendix	6 (0.5)	5:1
Cecum	258 (21.8)	153:105
Ascending colon	431 (36.5)	266:165
Transverse colon	124 (10.5)	78:46
Descending colon	149 (12.6)	102:47
Sigmoid colon	200 (16.9)	129:71
Rectosigmoid colon	4 (0.3)	1:3

The distribution of diverticula and the relationship between sex and location of diverticula were analyzed. When there was overlap in segments of the diverticula in one participant, all segments were evaluated in duplicate. The percentages of all participants are also listed.

(P = 0.236, P = 0.346 for trend), even among older patients. The presence of the bilateral type increased significantly with age (P < 0.001 for trend).

Relationship between diverticula and the length of the large intestine

There was no significant difference between the length of the large intestine without diverticula and that with diverticula (P = 0.104): median length was 157.2 cm (range: 104.9-259.3 cm) and 154.7 cm (range: 103.9-246.6 cm), respectively. The median length of the large intestine with ≥ 6 diverticula in cecum and/or ascending colon was 154.5 cm (range: 114.6-246.6 cm). This result also did not differ when compared to the length of the large intestine with no diverticula (P = 0.260). The median length of the large intestine with ≥ 6 and ≥ 21 diverticula in the sigmoid colon was 149.9 cm (range: 113.5-195.6 cm) and 145.1 cm (range: 115.0-192.0 cm), respectively, which were both significantly shorter than those with no diverticula (P < 0.001) (Table 4).

DISCUSSION

Geographical and racial variations have been reported in relation to the prevalence and distribution of colonic diverticula[1,4,17]. In developed Western countries, the prevalence of diverticulosis is high, affecting 5% of people in their 40s and approximately half of those in their 80s[18], with an estimated mortality rate of 2.5% per 100000 per year. This disease is the fifth most important gastrointestinal disease in terms of healthcare costs[19]. The incidence of diverticula has also been reported to increase over time[5,8]. The most recent large studies using colonoscopy showed that the prevalence of diverticulosis was 42% in the United States[4] and 23.9% in Japan[8]. Diverticula are mainly left-sided in patients in the United States and in other western countries. On the other hand, patients in Japan often manifest right-sided diverticula, with an associated age-related increase in disease prevalence[1,4,5,8,12]. In our study, the prevalence of colonic diverticulosis detected using CTC was 48.1%, which is much higher than the rates reported in previous studies using colonoscopy in Japan[5,8]. A report from Europe on the prevalence of diverticula diagnosed by CTC reported a prevalence of 51.6%, similar to ours, with a higher prevalence in the right-sided colon than expected[15]; however, compared to our results, the prevalence of diverticula in the right-sided colon in Caucasians was lower than that in Japanese. Most of the recent epidemiologic studies of diverticulosis have been evaluated through colonoscopy, which is a standard technique that is performed worldwide for the screening and diagnosis of colonic diseases[4,5,8]. However, investigators have reported that compared to colonoscopy, assessments evaluated using barium enema are superior for diagnosing colonic diverticulosis[13,14]. Among diverticula that had been detected using barium enema, Niikura *et al*[14] reported colonoscopy detection ratios of 41% and 32% for diverticula in the entire colon and in the left colon, respectively. Therefore, the actual prevalence of colonoscopy-detected diverticulosis in Japan may be over 25%. Conducting clinical studies on the prevalence of diverticulosis determined that the use of barium enema is difficult, given the widespread use of

Table 3 Sex and grade of diverticula in each segment and age group, *n* (%)

	Age group (yr)				
	≤ 40 (*n* = 108)	41-50 (*n* = 165)	51-60 (*n* = 268)	61-70 (*n* = 327)	≥ 71 (*n* = 313)
Sex					
Male	83(76.9)	98(59.4)	147 (54.9)	185 (56.6)	156 (49.8)
Female	25 (23.1)	67 (40.6)	121 (45.1)	142 (43.4)	157 (50.2)
Grade of diverticula in segment					
Ileum					
Rare	0	2 (1.2)	2 (0.7)	5 (1.5)	4 (1.3)
Multiple	0	0	0	0	0
Severe	0	0	0	0	0
Appendix					
Rare	0	0	1 (0.4)	4 (1.2)	1 (0.3)
Multiple	0	0	0	0	0
Severe	0	0	0	0	0
Cecum					
Rare	10 (9.3)	21 (12.7)	48 (17.9)	77 (23.5)	75 (24.0)
Multiple	0	3 (1.8)	9 (3.4)	7 (2.1)	4 (1.3)
Severe	0	0	1 (0.4)	3 (0.9)	0
Ascending colon					
Rare	12 (11.1)	35 (21.2)	60 (22.3)	81 (24.8)	77 (24.6)
Multiple	4 (3.7)	8 (4.8)	30 (11.2)	34 (10.4)	49 (15.7)
Severe	1 (0.9)	1 (0.6)	11 (4.1)	15 (4.6)	13 (4.2)
Transverse colon					
Rare	5 (4.6)	8 (4.8)	25 (9.3)	29 (8.9)	30 (9.6)
Multiple	0	0	4 (1.5)	12 (3.7)	9 (2.9)
Severe	0	1 (0.6)	0	1 (0.3)	0
Descending colon					
Rare	4 (3.7)	6 (3.6)	25 (9.3)	30 (9.2)	32 (10.2)
Multiple	0	2 (1.2)	8 (3.0)	14 (4.3)	14 (4.5)
Severe	0	1 (0.6)	0	5 (1.5)	8 (2.6)
Sigmoid colon					
Rare	7 (6.5)	7 (4.2)	29 (10.8)	31 (9.5)	34 (10.9)
Multiple	0	0	10 (3.7)	20 (6.1)	27 (8.6)
Severe	0	2 (1.2)	3 (1.1)	11 (3.4)	19 (6.1)
Rectosigmoid colon					
Rare	0	0	1 (0.4)	0	2 (0.6)
Multiple	0	0	0	0	1 (0.3)
Severe	0	0	0	0	0

In each colonic segment, the prevalence and the number of diverticula increased with age. Diverticula identified in the appendix, terminal ileum, and rectosigmoid colon were also evaluated. Rare: 1-5 diverticula; Multiple: 6-20 diverticula; Severe: ≥ 21 diverticula.

colonoscopy. According to the European Society of Gastrointestinal Endoscopy and the European Society of Gastrointestinal and Abdominal Radiology Guideline -

Table 4 Length of the large intestine by states of diverticula

	Median (cm)	Range (cm)
Diverticula negative in any segment	157.2	104.9-259.3
Diverticula positive in any segment	154.7	103.9-246.6
Multiple in cecum to ascending colon	154.5	114.9-246.6
Severe in cecum to ascending colon	151.6	119.6-198.4
Multiple in sigmoid colon	149.9	113.5-195.6
Severe in sigmoid colon	145.1	115.0-192.0

The large intestine of participants with multiple and severe diverticula in the sigmoid colon was significantly shorter than that of the participants with no diverticula ($P < 0.001$).

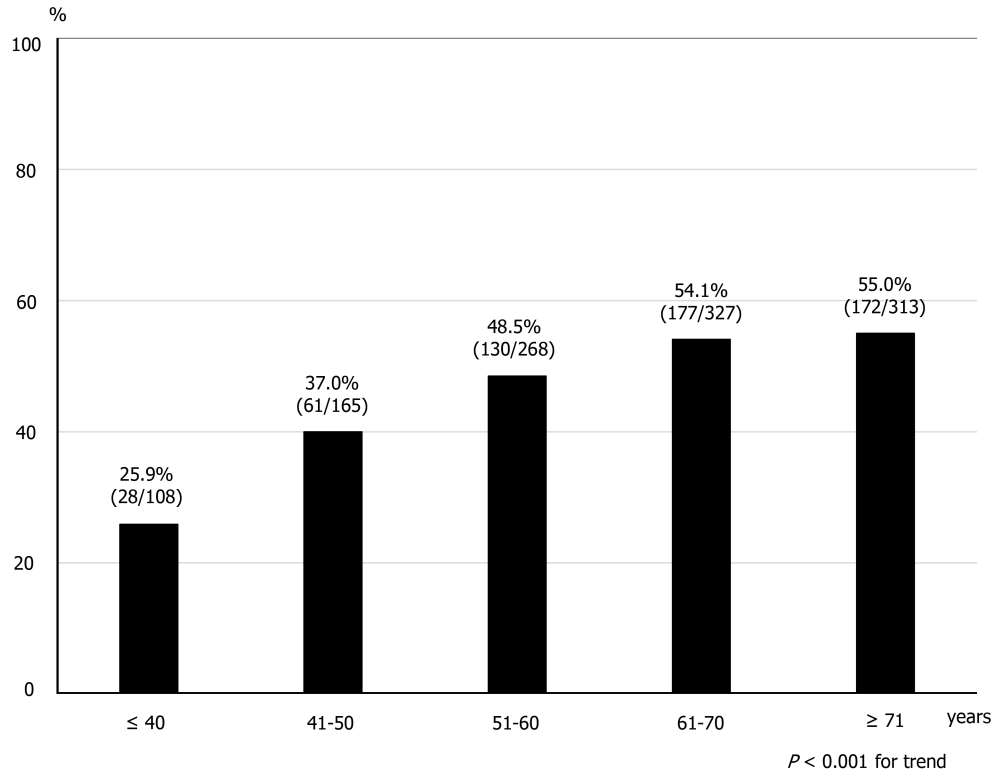


Figure 2 Prevalence of diverticulosis by age group. The prevalence of diverticulosis increased with age ($P < 0.001$ for trend).

Update 2020[20], barium enema for the diagnosis of colorectal neoplasia was not recommended (strong recommendation); in this regard, barium enema cannot be used in this setting.

In recent years, CTC has become more popular for screening and diagnosing colorectal cancer[21,22]; therefore, CTC could be used as an alternative to colonoscopy due to its diagnostic ability and safety[20,23]. It might be superior for detecting diverticula because it can evaluate the colon from more advantageous perspectives and avoid impediments caused by overlapping adjacent sections of the colon. In fact, De Cecco *et al*[15] reported that the prevalence of diverticulosis identified using CTC was 51.6%, which was much higher than that previously reported using colonoscopes in Western countries[4]. This result is similar to our results.

Our results showed that the prevalence of diverticulosis is increasing in Japan, mimicking the trend seen in Western countries. Several studies have shown that age, low intake of dietary fiber, fat intake, red meat intake, alcohol consumption, smoking, and increasing body weight are pathogenic risk factors associated with colonic diverticulosis[1,4,7,8,24]. Increasing age and changing to a westernized diet might be the most closely associated risk factor for the increasing prevalence of diverticulosis in

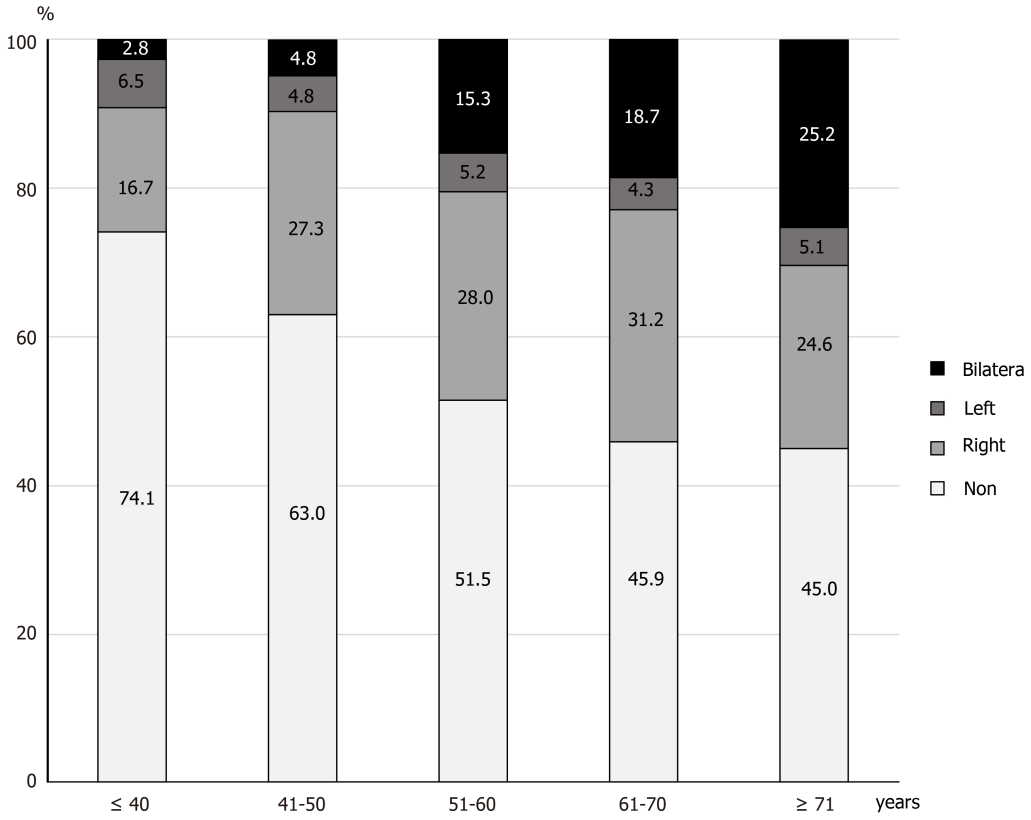


Figure 3 Location of diverticula by age group. An analysis of all participants showed that diverticulosis of the younger generation was predominantly right-sided, and bilateral diverticulosis increased with age ($P < 0.001$ for trend).

Japan.

In our study, the prevalence and grades of colonic diverticula increased with age, which concurs with the findings of previous studies[1,4,8]. Moreover, right-sided colonic diverticula were more predominant in patients aged < 70 years. While the prevalence of diverticula in the right- and left-sided colon did not differ, even in older patients, the prevalence of bilateral diverticula was higher in older patients. Patients with right-sided diverticula only may eventually develop left-sided diverticula with aging. Consequently, they may ultimately develop bilateral diverticula[25]. Although reports from Western countries suggest that the rate of diverticulosis does not differ between the sexes, our results showed that diverticulosis was more common in men, which is consistent with the findings of previous studies in Japan[1,4,5,8].

In this study, diverticula were also identified in the terminal ileum and appendix. Although there are few case reports and research on resected specimens[26-28], this is the first epidemiological report of asymptomatic diverticulosis of the appendix and terminal ileum. This should be considered in the differential diagnosis of acute abdomen and lower gastrointestinal bleeding. Diverticula in the appendix and terminal ileum, and as well as low numbers of right-sided diverticula, might be congenital. Similar to colonic diverticula, inflammation and hemorrhage are occasional consequences.

This is the first study to investigate the relationship between the length of the large intestine and the presence of diverticula. The large intestine with multiple (≥ 6) diverticula in the sigmoid colon was significantly shorter than that with no diverticula. There have been no reports of studies on the relationship between colonic diverticulosis and the length of the large intestine. The ability to accurately measure the length of the large intestine using a workstation is an advantage of CTC, a study made possible because of CTC. However, the length of the large intestine did not differ between the participants with multiple diverticula in the cecum and/or ascending colon in those with no diverticula. When multiple diverticula are present, the colonic wall can thicken and become rigid[18], and the sigmoid colon may shorten because it is free in the peritoneal cavity. In contrast, the colon is unlikely to shorten in patients with multiple right-sided diverticula because it is attached to the retroperitoneum.

This study had several limitations. First, about 70% of asymptomatic participants participated for screening purpose. The study also included participants who underwent CTC for the purpose of examination for any abdominal symptoms, or positive FIT, or abnormal blood test, or a familial history of CRC and polyps, or for the surveillance of polyps. Hence, the findings of this study may not necessarily reflect those of an asymptomatic general population. Second, participants aged > 50 years accounted for > 75% of the study population, and only a small number of patients aged < 40 years were included in this study. This differs from the age structure of the general population in Japan, which may explain why the prevalence of colonic diverticula in this study was much higher than what was previously reported. However, asymptomatic younger people have not been evaluated in previous studies. There might be the same bias in previous studies diagnosed by colonoscopy or barium enema on the prevalence of diverticula. Third, the patients' diet, lifestyle, and physical factors could underlie the increase in the prevalence of diverticulosis; however, data describing these factors were absent from this study. Fourth, the number of participants was smaller than the number of studies conducted based on colonoscopy [5,7,8]. This is because CTC is not yet widely used as a screening modality for colorectal neoplasms, and the facilities that can be used are limited. However, the number of participants in this study was slightly larger than that in a previous study conducted by CTC[15]. Moreover, the data used in this study are approximately 10 years old and are a bit out of date. We believe that a large-scale prospective study should be conducted after standardization of bowel preparation and imaging protocols in the future.

CONCLUSION

This is the first investigation to use CTC to evaluate the prevalence and distribution of colonic diverticula in Japan. The prevalence of colonic diverticulosis was higher than that reported previously. In addition to the superiority of CTC in detecting colonic diverticula, the aging of the Japanese population and the change to a Western diet may be behind the high prevalence of colonic diverticulosis observed in this study. The diverticula were predominantly right-sided in the younger generation, while right- and left-sided diverticula were more common in older participants. The prevalence of asymptomatic diverticulosis in the terminal ileum and appendix can be clarified for the first time. These results may be helpful in the management of diverticular diseases.

ARTICLE HIGHLIGHTS

Research background

Colonic diverticulosis is a common abnormality of the colon. The prevalence of diverticulosis and diverticular disease in Japan is increasing due to aging and a westernized diet.

Research motivation

Recent trends in the prevalence and distribution of colonic diverticula in Japan are not clear. Most recent studies on diverticulosis in Japan involved the use of colonoscopy. In recent years, computed tomography colonography (CTC) has been widely used as an alternative colon cancer screening method. We considered CTC to detect diverticula as outpouchings of the colonic wall from a more advantageous perspective, and it may be superior to colonoscopy and barium enema for detecting diverticula.

Research objectives

The main objective of this study was to evaluate the prevalence and distribution of colonic diverticula in Japan.

Research methods

This study included 1181 participants from 14 hospitals in Japan. We analyzed the prevalence and distribution of colonic diverticula and their relationships with age and sex using CTC and analyzed the relationship between the diverticula and the length of the large intestine.

Research results

Diverticulosis was present in 48.1% of the participants. The prevalence of diverticulosis was higher in the older participants. The diverticula seen in younger participants were predominantly located in the right-sided colon and on the bilateral side for older participants. The length of the large intestine with multiple diverticula in the sigmoid colon was significantly shorter in those without diverticula.

Research conclusions

The prevalence of colonic diverticulosis in Japan is 48.1%. This was much higher than that previously reported. In older participants, the prevalence was higher, and the distribution tended to be bilateral.

Research perspectives

The data used in this study were slightly limited and a bit out of date. We hope to perform a large-scale prospective study after standardization of bowel preparation and imaging protocols, and CTC use will become widespread in the future. We also hope to determine the relationship between the trends in diverticulosis and its accompanying symptoms.

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ORIGINAL ARTICLE

Observational Study

Prognostic role of plasma level of angiopoietin-1, angiopoietin-2, and vascular endothelial growth factor in hepatocellular carcinoma

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Abstract

BACKGROUND

Most hepatocellular carcinomas (HCCs) are hypervascular, with characteristic features of hepatic arterial supply to the tumor. The factors involved in tumor angiogenesis include angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), and vascular endothelial growth factor (VEGF).

AIM

To investigate the profiles of plasma levels of angiogenesis markers in patients with HCC and evaluate their roles in predicting overall survival (OS) and progression-free survival (PFS).

METHODS

Plasma samples from 240 prospectively enrolled HCC patients in the very early to advanced stages were used to measure the levels of Ang-1, Ang-2, and VEGF. Their associations with clinical characteristics, OS, and PFS were analyzed.

RESULTS

The median plasma levels of Ang-1, Ang-2, and VEGF were 3216 pg/mL, 1684 pg/mL, and 26.5 pg/mL, respectively. The plasma level of Ang-2 showed a significant increase from early stage [Barcelona clinic liver cancer (BCLC) A] to intermediate (BCLC B) and advanced stage HCC (BCLC C/D), whereas Ang-1, VEGF, and alpha-fetoprotein (AFP) levels in the plasma did not show any such changes. Multivariable analysis, propensity score-matched analysis, and time-dependent receiver operating curve analysis revealed that Ang-2 levels had the highest predictive power for OS and PFS. Neither Ang-1 nor VEGF was significantly associated with OS or PFS. The neutrophil-to-lymphocyte ratio was an independent factor for OS and PFS.

data.

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CONCLUSION

The plasma levels of Ang-2 correlated with liver function, tumor stage, and tumor invasiveness, showing better performance in predicting OS and PFS than AFP, Ang-1, or VEGF.

Key Words: Hepatocellular carcinoma; Angiogenesis; Biomarker; Prognosis; Survival

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Core Tip: Most hepatocellular carcinomas (HCCs) are hypervascular tumor, thus angiogenesis markers can be a potential biomarker. This study explored the potential of each plasma level of angiopoietin (Ang)-1, Ang-2, and vascular endothelial growth factor as a prognostic biomarker for very early to advanced stages of HCC *via* detailed analysis in comparison with alpha-fetoprotein. The plasma level of Ang-2 correlated with liver function, tumor stage, and tumor invasiveness. Multivariable and propensity score-matched analyses revealed Ang-2 levels with the highest predictive power for overall survival in patients with HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most common malignancy in men, and seventh in women, and the second leading cause of cancer-related deaths worldwide [1]. Most HCCs are hypervascular with characteristic features of hepatic arterial supply to the tumor, frequent portal vein invasion, and intrahepatic metastasis. Based on these characteristics, the diagnosis of HCC based on radiological criteria was established to minimize the bleeding and seeding complications of tumor biopsy in most cases.

Therapeutically, transarterial chemoembolization (TACE), a standard treatment modality for the intermediate stage of HCC, uses the characteristic tumor blood supply for the selective arterial delivery of chemotherapeutic agents and the blockade of tumor-supplying vessels with embolic material. In advanced-stage HCC, several targeted therapeutics, including sorafenib, regorafenib, lenvatinib, ramucirumab, and cabozantinib, inhibit the angiogenic pathway along with other signaling pathways[2-4]. Recently, the combination of an anti-angiogenic agent and an immune checkpoint inhibitor showed a superior effect to sorafenib in the treatment of advanced HCC[5]. Therefore, the development of biomarkers for angiogenesis to assess treatment response and prognosis for HCC is becoming important.

Angiogenesis, the formation of new blood vessels from pre-existing ones, is a fundamental process in HCC development, progression, and metastasis[6,7]. In contrast to normal vessels, tumor vessels have highly proliferative endothelial cells with a leaky vasculature, which is unable to provide oxygen and nutrient supply to the tumor, leading to aberrant tumor microenvironments. The factors involved in tumor angiogenesis include vascular endothelial growth factor (VEGF), angiopoietin (Ang), fibroblast growth factor, epidermal growth factor, insulin-like growth factor, and platelet-derived growth factor. This makes the tumor angiogenesis system highly complex[8]. Among these, VEGF and Ang-tyrosine kinase with Ig and EGF homology domains-2 (Tie2) pathways, including Ang-1 and Ang-2[7,9,10], are the two dominant therapeutic targets of anti-angiogenesis in HCC.

However, investigations on the prognostic value of blood angiogenesis biomarkers across all HCC stages till date are limited. Therefore, this study aimed to investigate the plasma levels of Ang-1, Ang-2, and VEGF, and to evaluate their roles in predicting the overall survival (OS) and progression-free survival (PFS) in patients with very early to advanced stages of HCC.

MATERIALS AND METHODS

Study subjects

A total of 251 newly diagnosed HCC patients who agreed to the collection of their information and blood samples at the time of diagnosis were prospectively enrolled in the Hepatology unit of the Seoul National University Bundang Hospital (SNUBH) between March 2012 and April 2016 (Supplementary Figure 1). Patients with extrahepatic malignancies that might affect survival (n = 9) and those with combined hepatocellular cholangiocarcinoma (n = 2) were excluded accordingly. The final HCC group hence, included 240 patients. HCC diagnosis was based on histologic examination and/or typical features (nodules ≥ 1 cm with arterial hypervascularity and portal or delayed washout) on dynamic computed tomography and/or magnetic resonance imaging[11]. Written informed consent was obtained from each HCC patient after approval by the Institutional Review Board (IRB) of the SNUBH. (IRB No. B-1201/143-002)

Data collection and follow-up of patients with HCC

Clinical and pathological data of the HCC group were collected from electronic medical records. Data included age, sex, height, weight, etiology of HCC, comorbidities, complete blood cell count, neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), blood levels of alpha-fetoprotein (AFP), serum aspartate aminotransferase, alanine aminotransferase, albumin, total bilirubin, prothrombin time expressed as the international normalized ratio, creatinine, Child-Pugh score and class, model for end-stage liver disease (MELD) score, tumor size, tumor number, tumor distribution, presence of macrovascular invasion and/or distant metastasis, tumor-node metastasis (TNM) stage based on the American Joint Committee on Cancer, 8th edition[12], and Barcelona clinic liver cancer (BCLC) stage[2,3].

The primary outcome was OS, and the secondary outcome was the PFS. The index date was defined as the date when patients were diagnosed with HCC on their first liver protocol computed tomography or magnetic resonance imaging. The HCC patients were followed up until December 2019 for a median duration of 3.6 years. The mortality of each patient was confirmed by the requested data from Statistics Korea, and the cause of death was checked with the help of their medical records. PFS was estimated as the interval between the date of diagnosis and the date of progression confirmed on imaging study, death, or the end of follow-up.

Measurement of plasma levels of Ang-1, Ang-2, and VEGF in HCC patients

Blood samples were collected from patients with HCC before treatment. The collected samples were centrifuged at 4000 × g for 10 min to obtain plasma and stored at −70 °C until use. Plasma levels of Ang-1, Ang-2, and VEGF were measured using a commercial enzyme-linked immunoassay (Luminex® screening and performance assays, R&D systems, Minneapolis, MN, United States) according to the manufacturer's instructions. All samples were analyzed at least twice.

Statistical analysis

The baseline characteristics of the patients were compared using the chi-square test for categorical variables, and a t-test was used to compare continuous variables. Spearman rank correlation was used to calculate the correlation coefficient between the ranked variables. OS and PFS were estimated using the Kaplan–Meier method and compared using the log-rank test. The Cox proportional hazards model was employed to determine the hazard ratio (HR) of OS and PFS in the univariate and subsequent multivariate analyses.

The prognostic values of the plasma biomarkers were determined by time-dependent receiver operating characteristic (ROC) curve analysis from censored survival data using the nearest-neighbor estimation method. All P values were two-sided, and values < 0.05, were considered significant. SPSS (version 21; SPSS, Inc., Chicago, IL, United States) and R (version 3.6.3., http://cran.r-project.org/) software was used for the statistical analyses.

To confirm the prognostic value of Ang-2 by reducing the impact of potential confounding effects, significant differences in baseline characteristics between the high and low Ang-2 groups were adjusted by propensity score (PS) matching. We used nearest-neighbor matching with a caliper size of 0.1, and matched the patients in a 1:1 ratio.

RESULTS

Baseline characteristics and plasma levels of Ang-1, Ang-2, VEGF, and AFP in patients with HCC

Baseline characteristics of the subjects are presented in Table 1. The 240 HCC patients had a mean age of 60.9 years, and there was a male proportion of 80.4%. The etiologies of HCC were as follows: Hepatitis B virus (HBV) (69.6%), hepatitis C virus (10.0%), alcohol (11.7%), and others (7.9%). The HCC group included patients with Child-Pugh class B or C (14.9%), TNM stage I or II (65.5%), and BCLC stage 0 or A (55.5%) (Table 1). There were no missing values for the variables.

The median plasma levels of Ang-1, Ang-2, VEGF, and AFP were 3216 pg/mL, 1684 pg/mL, 26.5 pg/mL, and 17.9 ng/mL, respectively. All biomarkers showed an increasing trend as the BCLC stage progressed (Figure 1). However, only the median plasma level of Ang-2 showed a significant increase from the early stage HCC (BCLC A: 1289 pg/mL) to intermediate (BCLC B; 1808 pg/mL) and advanced stage HCC (BCLC C/D; 3653 pg/mL), whereas Ang-1, VEGF, and AFP levels did not show any changes. Ang-2 and AFP levels showed an increasing trend as the TNM stage progressed, while Ang-1 and VEGF levels did not (Supplementary Figure 2). NLR and PLR showed an increasing trend as the BCLC stage progressed (Supplementary Figure 3).

Factors correlated with plasma levels of Ang-1, Ang-2, and VEGF in HCC patients with their interrelationship

The correlation plot between each plasma level of the three biomarkers and clinical factors is shown in Figure 2. Ang-2 levels were significantly correlated with tumor extent and poor liver function, while VEGF levels were correlated with tumor extent. Meanwhile, the rho value between Ang-1 or VEGF levels and liver function or tumor extent was not significant. Ang-2 was significantly correlated with both NLR and PLR, while Ang-1 and VEGF were correlated with PLR only. Ang-2 levels were not correlated with VEGF, but negatively correlated with Ang-1 levels. Meanwhile, Ang-1 was positively correlated with VEGF levels in the HCC group (Figure 2).

The median plasma levels of Ang-2 and VEGF did not differ according to the etiology of HCC (Supplementary Figure 4A and B). The correlation plot between each plasma level of the biomarkers and HBV DNA is shown in Supplementary 4C and D among CHB patients (n = 156). HBV DNA levels were not significantly correlated with either Ang-2 or VEGF levels.

The comparison of the clinical factors between high- and low-level groups of Ang-2 or VEGF using the median level as a cutoff is shown in Supplementary Table 1. The high Ang-2 and high VEGF groups showed more advanced tumor stages than the low Ang-2 and low VEGF groups. The high Ang-2 group had a significantly higher proportion of Child-Pugh class B or C than the low Ang-2 group (26.1% *vs* 4.1%, P < 0.001). On the contrary, the high VEGF group showed a significantly lower MELD score than the low VEGF group (3.8 *vs* 5.6, P = 0.01). The NLR and PLR of the high Ang-2 group were significantly higher than those of the low Ang-2 group (3.5 *vs* 2.2, P = 0.007; 138.3 *vs* 98.5. P = 0.01). However, there was no difference in the NLR and PLR between the high and low VEGF groups.

Potential of the plasma levels of Ang-1, Ang-2, or VEGF as prognostic markers for HCC compared to AFP level in the aspect of OS

During a median follow-up of 3.6 years, 118 deaths were observed in the HCC group. The OS curves between the low and high levels of Ang-1, Ang-2, VEGF, and AFP using the median level as a cutoff are shown in Figure 3. The OS was significantly shorter in the high Ang-2 group [HR 4.76, 95% confidence interval (CI): 3.15–7.19, P < 0.001; Figure 3B] and high AFP group (≥20 ng/mL) (HR 2.97, 95%CI: 2.02–4.37, P < 0.001; Figure 3D) than in the low Ang-2 and low AFP groups, respectively. However, the OS rate was not significantly different between the high and low Ang-1 groups (HR 1.02, 95%CI: 0.71-1.56, P = 0.82; Figure 3A) and between the high and low VEGF groups (HR 1.39, 95%CI: 0.97–2.00, P = 0.07; Figure 3C).

The results of the univariate and multivariate analyses for the factors associated with OS are shown in Table 2. The high Ang-2 group was an independent factor (HR 5.96, 95%CI: 1.58–22.43, P < 0.001) associated with OS, along with NLR (HR 4.45, 95%CI: 1.77–11.23, P = 0.002), Child-Pugh class B or C, and TNM stage. Meanwhile, VEGF and AFP levels were not associated with OS (Table 2).

Table 1 Baseline characteristics and plasma levels of angiopoietin-1, angiopoietin-2, and vascular endothelial growth factor

Characteristic	HCC patients (*n* = 240)
Age, mean age ± SD, year	60.9 ± 11.2
Male sex, *n* (%)	193 (80.4)
BMI, mean ± SD, kg/m^2	24.1 ± 3.0
Etiology, *n* (%)	
HBV/HCV/HBV + HCV	167 (69.6)/24 (10)/2 (0.8)
Alcohol/Others	28 (11.7)/19 (7.9)
Presence of cirrhosis, *n* (%)	184 (76.7)
Child-Pugh score, *n* (%)	
A/B/C	204 (85.1)/35 (14.5)/1 (0.4)
ECOG performance status, *n* (%)	
0/1–2/3	156 (65)/80 (33.3)/4 (1.7)
Maximal tumor size, median (IQR), cm	3.6 (2.0–7.0)
Multi-nodularity of tumor, *n* (%)	105 (43.8)
Vascular invasion of HCC, *n* (%)	45 (18.8)
Presence of distant metastasis, *n* (%)	18 (7.5)
BCLC staging, *n* (%)	
0/A	52 (21.7)/81 (33.8)
B/C/D	43 (17.9)/59 (24.6)/5 (2.1)
TNM staging, *n* (%)	
I/II	115 (48.0)/42 (17.5)
III/IV	65 (27.1)/18 (7.5)
Initial treatment, *n* (%)	
Resection	34 (14.2)
RFA/TACE	27 (11.3)/165 (69.2)
Sorafenib/BSC	2 (0.8)/11 (4.6)
Laboratory results	
WBC, median (IQR), × 10^3/uL	5.1 (4.0–6.6)
Hemoglobin, median (IQR), g/dL	13.6 (12.0–14.8)
Platelet count, median (IQR), × 10^9/uL	130 (88–184)
Prothrombin time, median (IQR), INR	1.11 (1.04–1.18)
AST, median (IQR), IU/mL	45 (32–73)
ALT, median (IQR), IU/mL	38 (23–61)
Albumin; median (IQR), g/dL	4.0 (3.5–4.2)
Total bilirubin, median (IQR), mg/dL	0.8 (0.5–1.1)
Creatinine, median (IQR), mg/dL	0.8 (0.7–1.0)
AFP, median (IQR), ng/mL	17.9 (4.0–698)
Angiogenesis marker	
Ang-1, median (IQR), pg/mL	3216 (1565–6266)
Ang-2, median (IQR), pg/mL	1684 (1107–3064)
Ang-2/Ang-1 ratio, median (IQR)	0.56 (0.25–1.39)
VEGF, median (IQR), pg/mL	26.5 (13.8–51.3)

NLR, mean ± SD	2.8 ± 3.5
PLR, mean ± SD	118.2 ± 119.7

AFP: α-feto protein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; BCLC: Barcelona clinic liver cancer; BSC: Best supportive care; ECOG: Eastern Cooperative Oncology Group; HCC: Hepatocellular carcinoma; IQR: Interquartile range; NLR: Neutrophil-lymphocyte ratio; SD: Standard deviation; TNM: Tumor-node-metastasis; PLR: Platelet-lymphocyte ratio; VEGF: Vascular endothelial growth factor; WBC: White blood cell.

Table 2 Univariate and multivariate analysis on the factors predictive for poor overall survival in hepatocellular carcinoma patients

Variable	Univariate analysis		Multivariable analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Ang-2 group (cut-off: Median level)				
Low Ang-2 group	Reference	–	Reference	–
High Ang-2 group	4.76 (3.15 – 7.20)	< 0.001	5.96 (1.58–22.43)	< 0.001
VEGF group (cut-off: Median level)				
Low VEGF group	Reference	–	Reference	–
High VEGF group	1.39 (0.97–2.00)	0.07	0.80 (0.33–1.98)	0.63
Ang-2/Ang-1 ratio	1.02 (0.94–1.06)	0.26	–	–
AFP (≥ 20 ng/mL)	2.97 (2.02–4.37)	< 0.001	2.63 (0.85–8.09)	0.09
Age	1.00 (0.98–1.02)	0.93	–	–
Male sex	1.05 (0.68–1.64)	0.82	–	–
BMI > 25 kg/m^2	0.70 (0.47–1.03)	0.07	–	–
Presence of cirrhosis	1.02 (0.67–1.57)	0.92	–	–
Child-Pugh class B or C	3.68 (2.42–5.58)	< 0.001	5.59 (2.44–12.81)	< 0.001
NLR	3.24 (1.49–7.06)	0.003	4.45 (1.77–11.23)	0.002
PLR	2.68 (1.26–5.70)	0.01	0.56 (0.24–1.33)	0.19
TNM stage				
I	Reference	–	Reference	–
II	6.30 (1.15–34.42)	0.03	3.61 (0.62–21.08)	0.15
III	26.43 (6.01–116.24)	< 0.001	14.20 (2.82–71.45)	0.001
IV	70.42 (15.00–330.6)	< 0.001	41.39 (6.62–258.67)	< 0.001

Cox proportional hazards model with a backward elimination approach was used for multivariable analysis. HR: Hazard ratio; CI: Confidence interval; VEGF: Vascular endothelial growth factor; AFP: Alpha fetoprotein; BMI: Body mass index; MELD: Model for end stage liver disease; HCC: Hepatocellular carcinoma; TNM: Tumor-node-metastasis.

To analyze the survival prediction power of Ang-2 levels, time-dependent area under the ROC (AUROC) analysis for survival prediction showed that the AUROC of baseline Ang-2 levels (0.909) for the 1-year survival prediction was significantly higher than that of AFP (AUROC 0.817, $P = 0.03$), Ang-1 (AUROC 0.535, $P < 0.001$), and VEGF (AUROC 0.577, $P < 0.001$; Figure 4). The AUROC of Ang-2 (0.873) for the two-year survival prediction was also significantly higher than that of AFP (AUROC 0.767, $P = 0.01$), Ang-1 (AUROC 0.541, $P < 0.001$), and VEGF (AUROC 0.581, $P < 0.001$). Moreover, baseline Ang-2 levels showed better survival predictive power than the other plasma biomarkers throughout the 5 years (Figure 4).

PS-matching yielded 37 matched pairs of patients from the high Ang-2 and low Ang-2 groups (Supplementary Table 2). Within this matched cohort, there were no significant between-group differences in most baseline characteristics, except for the proportion of male patients (75.8% *vs* 83.8%). The high Ang-2 group showed a significantly higher risk of death (HR 2.29, 95%CI: 1.10–4.76, $P = 0.03$) than the low

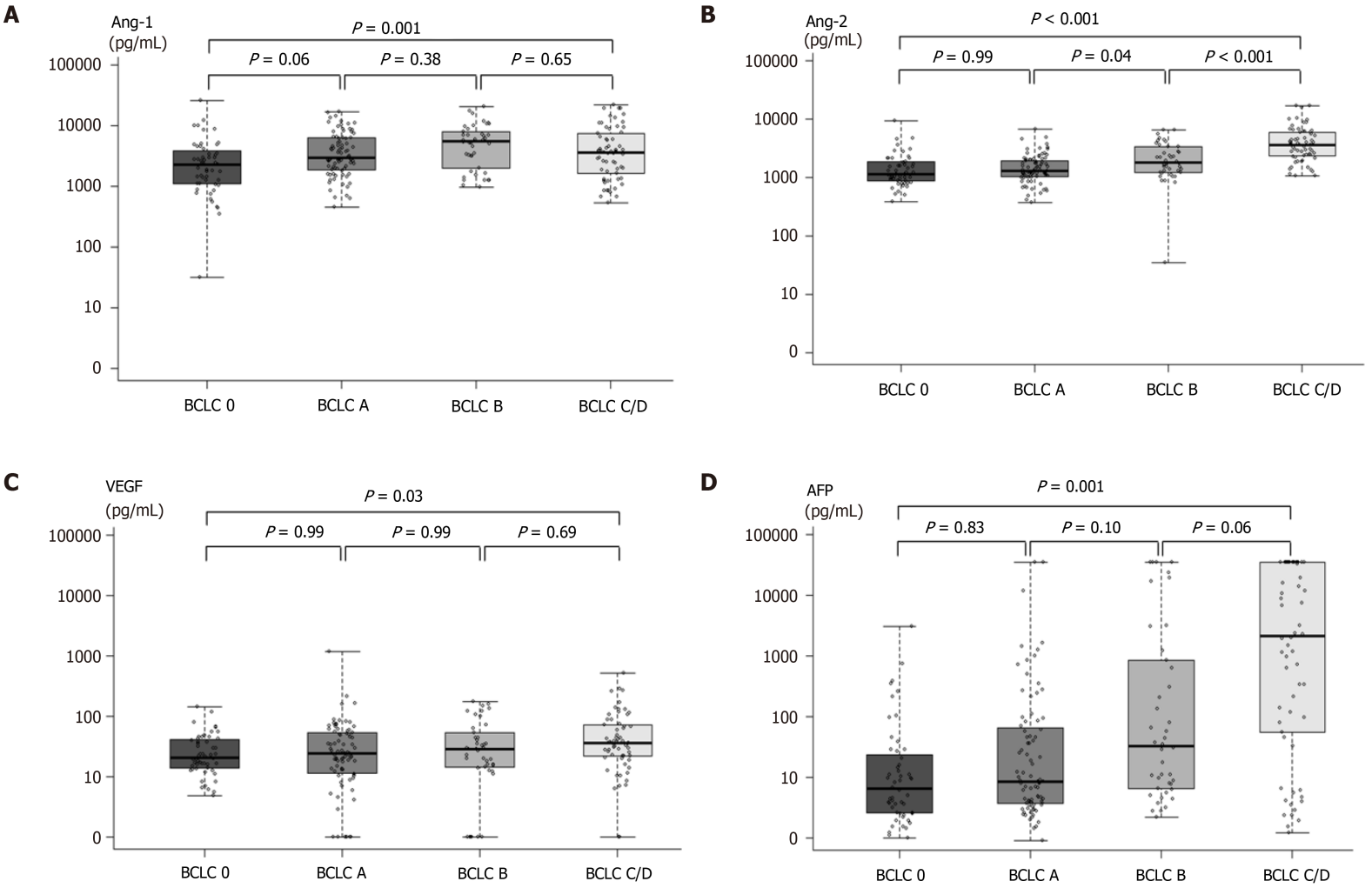


Figure 1 Plasma levels of angiopoietin-1, angiopoietin-2, vascular endothelial growth factor, and alpha fetoprotein in the hepatocellular carcinoma patients according to tumor stage. A: Angiopoietin-1; B: Angiopoietin-2; C: Vascular endothelial growth factor; D: Alpha-fetoprotein. AFP: Alpha-fetoprotein; Ang-1: Angiopoietin-1; Ang-2: Angiopoietin-2; BCLC: Barcelona clinic liver cancer; VEGF: Vascular endothelial growth factor.

Ang-2 group (Supplementary Figure 5A).

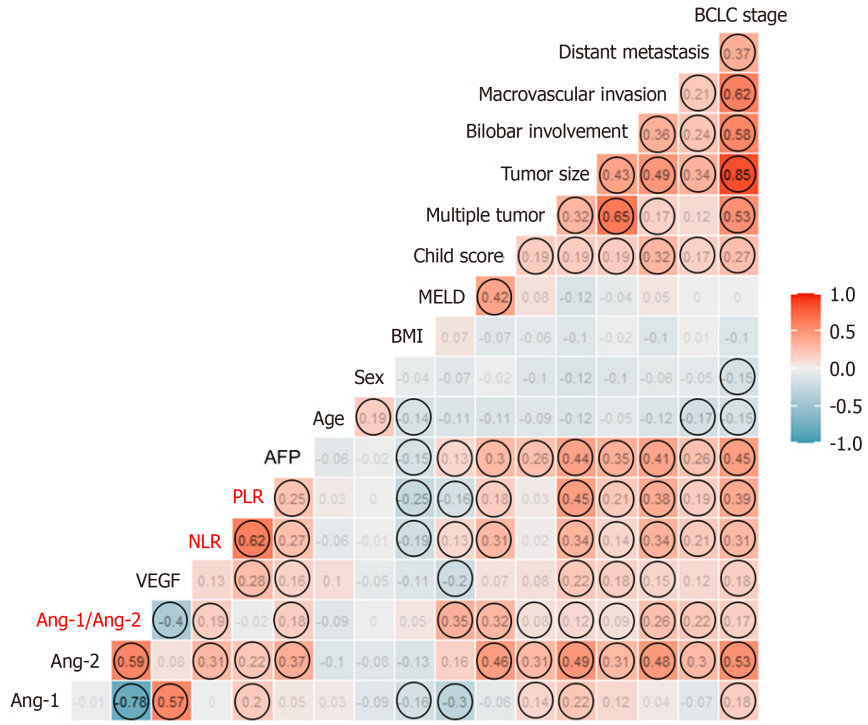


Figure 2 Factors correlated with plasma level of angiopoietin-1, angiopoietin-2 and vascular endothelial growth factor in hepatocellular carcinomas patients with their interrelationship. The heat map showing a red background indicates a positive correlation, the blue background indicates negative correlation, and the numbers in the box indicate rho (ρ). AFP: Alpha-fetoprotein; Ang-1: Angiopoietin-1; Ang-2: Angiopoietin-2; BCLC: Barcelona clinic liver cancer; BMI: Body mass index; MELD: Model for end stage liver disease; NLR: Neutrophil-lymphocyte ratio; PLR: Platelet-lymphocyte ratio; VEGF: Vascular endothelial growth factor.

Potential of the plasma levels of Ang-1, Ang-2, or VEGF as prognostic markers for HCC compared to AFP level in terms of PFS

During the median follow-up period, 171 progressions and 18 deaths were observed in the HCC group. The PFS curve between low and high levels of Ang-1, Ang-2, VEGF, and AFP using the median level as a cutoff are shown in Figure 5. The PFS was significantly shorter in the high Ang-2 group (HR 2.53, 95%CI: 1.89–3.39, $P < 0.001$; Figure 5B) and high AFP group (≥ 20 ng/mL) (HR 2.27, 95%CI: 1.71–3.04, $P < 0.001$; Figure 5D) than in the low Ang-2 and low AFP groups, respectively. However, the PFS rate was not significantly different between the high and low Ang-1 groups (HR 1.31, 95%CI: 0.99–1.74, $P = 0.06$; Figure 5A) or between the high and low VEGF groups (HR 1.29, 95%CI: 0.97–1.71, $P = 0.08$; Figure 5C).

The results of univariate and multivariate analyses for the factors associated with PFS are shown in Table 3. The high Ang-2 group was an independent factor (HR 1.55, 95%CI: 1.10–2.20; $P = 0.01$) associated with PFS, along with NLR (HR 1.95, 95%CI: 1.23–3.08, $P = 0.004$), AFP (HR 1.54, 95%CI: 1.13–2.11, $P = 0.007$), Child-Pugh class B or C, BMI, and TNM stage. However, VEGF and PLR were not (Table 3).

Within 37 pairs of PS-matched cohorts, PFS was not significantly different between the high and low Ang-2 groups (HR 1.19, 95%CI: 0.72–1.96, $P = 0.51$; Supplementary Figure 5B). This is probably due to the small sample size of PFS during follow-up.

DISCUSSION

This study explored the potential of each plasma level of Ang-1, Ang-2, and VEGF as a prognostic biomarker for very early to advanced stages of HCC *via* detailed analysis in comparison with AFP. Multivariable and PS-matched analyses revealed Ang-2 levels with the highest predictive power for OS in patients with HCC. Moreover, Ang-2 and AFP levels were independent factors for PFS. In contrast, neither Ang-1 nor VEGF was significantly associated with OS or PFS. NLR was an independent factor for both OS and PFS.

Table 3 Univariate and multivariate analysis on the factors predictive for progression-free survival in hepatocellular carcinoma patients

Variable	Univariate analysis		Multivariable analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Ang-2 group (cut-off: Median level)				
Low Ang-2 group	Reference	–	Reference	–
High Ang-2 group	2.53 (1.89–3.39)	< 0.001	1.55 (1.10–2.20)	0.01
VEGF group (cut-off: Median level)				
Low VEGF group	Reference	–	Reference	–
High VEGF group	1.29 (0.97–1.71)	0.08	1.08 (0.80–1.46)	0.61
Ang-2/Ang-1 ratio	1.02 (0.98–1.05)	0.32	–	–
AFP (≥ 20 ng/mL)	2.27 (1.713.04)	< 0.001	1.54 (1.13–2.11)	0.007
Age	0.99 (0.98–1.01)	0.30	–	–
Male sex	1.32 (0.91–1.92)	0.15	–	–
BMI > 25 kg/m^2	0.67 (0.50–0.91)	0.01	0.72 (0.53–0.97)	0.03
Presence of cirrhosis	1.04 (0.74–1.46)	0.81	–	–
Child-Pugh class B or C	2.17 (1.48–3.18)	< 0.001	1.54 (1.01–2.35)	0.05
NLR > 4	2.13 (1.45–3.12)	< 0.001	1.95 (1.23–3.08)	0.004
PLR > 150	2.05 (1.45–2.90)	< 0.001	1.02 (0.67–1.57)	0.93
TNM stage				
I	Reference	–	Reference	–
II	1.94 (1.31–2.89)	0.001	1.75 (1.16–2.63)	0.008
III	5.47 (3.81–116.24)	< 0.001	4.35 (2.91–6.50)	< 0.001
IV	70.42 (15.00–330.6)	< 0.001	9.35 (5.00–17.46)	< 0.001

Cox proportional hazards model with a backward elimination approach was used for multivariable analysis. HR: Hazard ratio; CI: Confidence interval; VEGF: Vascular endothelial growth factor; AFP: Alpha fetoprotein; BMI: Body mass index; MELD: Model for end stage liver disease; HCC: Hepatocellular carcinoma; TNM: Tumor-node-metastasis.

In this study, the median levels of Ang-1, Ang-2, and VEGF were lower than those in previous reports that included only patients with advanced-stage HCC, mostly treated with sorafenib. This may be due to the relatively low proportion of advanced-stage HCC cases included in this study (27%). Ang-2 levels were higher in patients with advanced HCC than in those with early HCC. Moreover, the majority of our patients underwent locoregional therapy (radiofrequency ablation, 11.3%; TACE, 69.2%). Ang-2 Levels showed a positive correlation with liver function indicators, such as MELD score and Child-Pugh score, and tumor extent or aggressiveness, which are the two major axes of HCC prognosis. Similarly, a recent study reported that Ang-2 levels were associated with 90-d mortality, acute kidney injury stage, and risk of renal replacement therapy in a cohort of patients with decompensated cirrhosis[13]. Meanwhile, Ang-1 and VEGF levels were generally associated with tumor extent rather than liver function. Therefore, this study suggests that Ang-2 levels are better prognostic biomarkers for HCC than Ang-1 or VEGF levels, especially after locoregional treatment.

The potential of Ang-2 as a prognostic marker was confirmed by survival analysis. Our multivariable analysis showed that the plasma level of Ang-2 (median 1684 pg/mL), but not of VEGF (median 26.5 pg/mL), was an independent predictor of OS. In addition, time-dependent ROC curve analysis also demonstrated that Ang-2 level is a better predictor of OS than AFP, Ang-1, and VEGF levels, which was supported by PS-matched analysis.

Several studies have investigated the prognostic role of Ang-2 in patients with advanced HCC. In 2013, Miyahara *et al*[14] investigated eight cytokine levels in 120 patients with advanced HCC who were treated with sorafenib. The results showed that the Ang-2 level (median, 721.3 pg/mL) was associated with OS and PFS, but

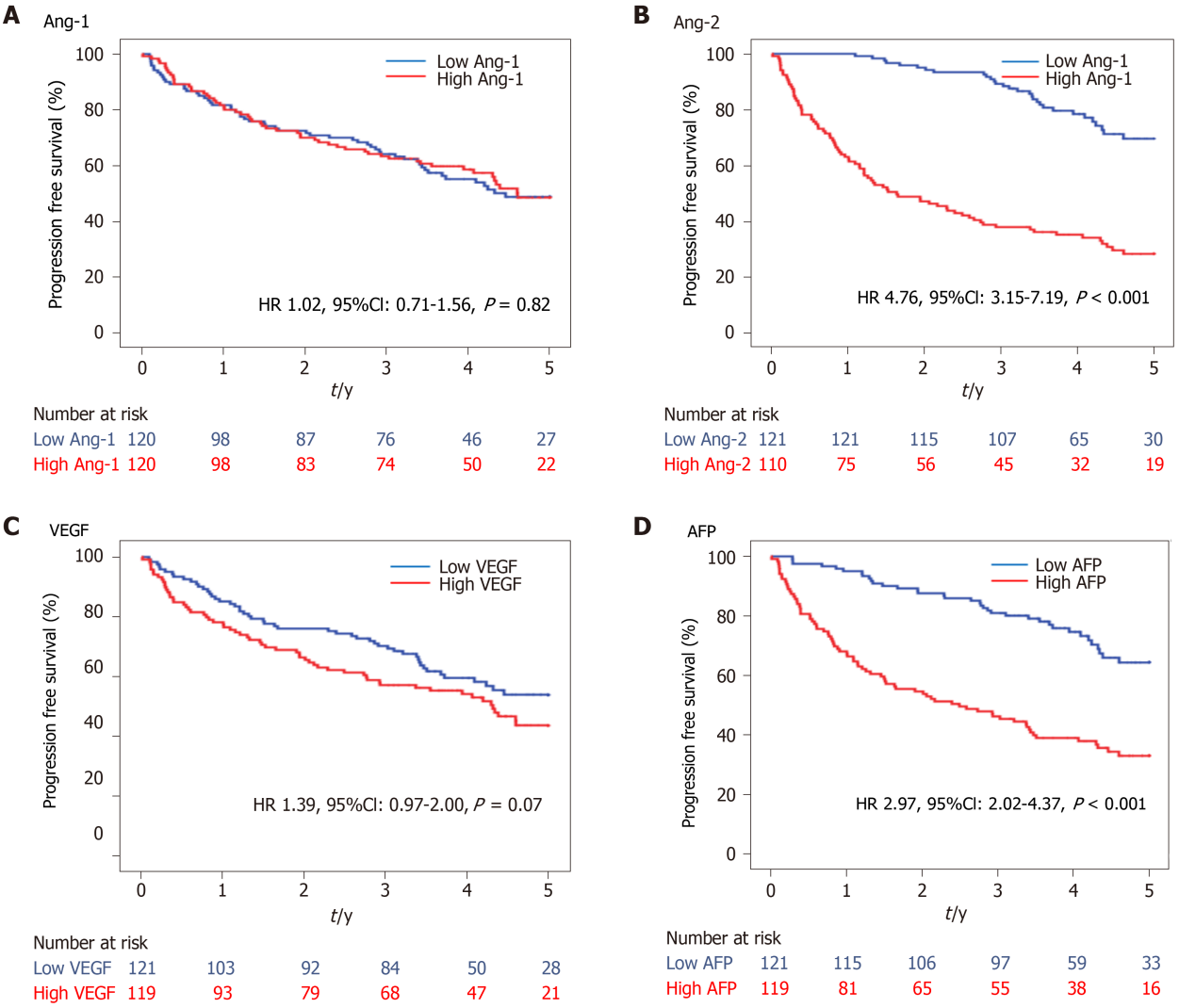


Figure 3 Overall survival according to plasma angiogenesis marker level. A: Angiopoietin-1; B: Angiopoietin-2; C: Vascular endothelial growth factor; D: Alpha-fetoprotein. AFP: Alpha-fetoprotein; Ang-1: Angiopoietin-1; Ang-2: Angiopoietin-2; VEGF: Vascular endothelial growth factor; HR: Hazard ratio; CI: Confidence interval.

VEGF (median 68.6 pg/mL) was not[14]. This result is consistent with that of our study. However, in our study, the median levels of Ang-2 (1684 pg/mL) and VEGF (26.5 pg/mL) were different. Llovet *et al*[15] reported that the plasma levels of Ang-2 (median, 6043.5 pg/mL) and VEGF (median, 1019 pg/mL) were independent predictors of OS in 602 advanced HCC enrolled in the phase 3, randomized controlled sorafenib HCC assessment randomized protocol (SHARP) trial, both of which were not significant response-prediction biomarkers in the sorafenib-treated cohort[15].

In a Chinese study of 173 HCC patients, Ang-2 level (mean, 18000 pg/mL) was an independent prognostic factor with a cutoff level of 6433 pg/mL[16]. Pestana *et al*[6] reported that a high plasma Ang-2 level (above mean value of 15300 pg/mL) was associated with lower OS, but a high Ang-1 level (above mean value of 16000 pg/mL) was associated with longer OS in 767 HCC patients treated at MD Anderson Cancer Center (77% in advanced stage)[6]. In this study, the Ang-1 and Ang-2 levels were markedly higher than that of ours and of previous studies because they showed mean rather than median values and used a different ELISA kit. A small-scale study in Spain (n = 33) showed that Ang-2 levels in peripheral blood and hepatic veins were well correlated (r = 0.95), and serum Ang-2 levels were significantly associated with tumor extent and aggressiveness. However, it was not related to treatment response.

VEGF is secreted by HCC cells and enhances endothelial cell survival, proliferation, invasion, and migration in response to tumor hypoxia[9]. Ang-1 and Ang-2 are both secreted proteins with 60% amino acid sequence homology, and they interact with Tie2, the endothelial cell-specific tyrosine kinase receptor[10]. While Ang-1 binds Tie2 to promote vessel stability and quiescence, Ang-2 binds to Tie2 to promote vessel

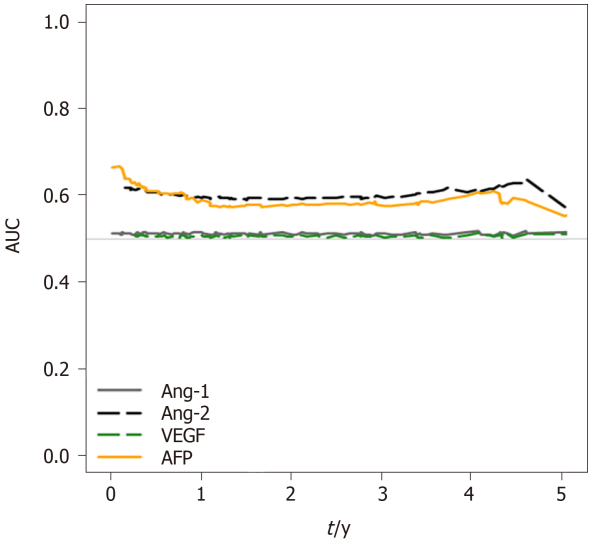


Figure 4 Time-dependent receiver operating characteristic for survival at each time point. AFP: Alpha-fetoprotein; Ang-1: Angiopoietin-1; Ang-2: Angiopoietin-2; VEGF: Vascular endothelial growth factor; ROC: Receiver operating characteristic; AUC: Area under the ROC curve.

permeability, instability, and eventual tissue hypoxia. This antagonistic relationship was consistent with the negative correlation between the plasma levels of Ang-1 and Ang-2 in our study. Angiogenic switch by Ang-2 promotes VEGF expression and promotes tumor angiogenesis.

On the other hand, Ang-2 acts as a chemoattractant by promoting recruitment of proangiogenic myeloid cells, especially Tie2 expressing macrophages (TEMs)[17,18]. TEMs become more proangiogenic and immunosuppressive in the tumor microenvironment. This is a potential mechanism for resistance to antiangiogenic therapy and poor survival in patients with high Ang-2 expression in HCC[19]. Interestingly, NLR and PLR, both inflammatory biomarkers, were significantly correlated with Ang-2 levels in this study. A recent meta-analysis showed that preoperative NLR was positively correlated with the risk of microvascular invasion in HCC[20]. Moreover, many studies have confirmed that a high baseline NLR is an independent factor for OS and PFS[21-23]. This is consistent with the results of our study.

Angiogenesis inhibitors have become an important therapeutic approach for the treatment of solid cancers. First-generation anti-angiogenic agents, including bevacizumab, sorafenib, pazopanib, vandeltanib, cabozantinib, axitinib, VEGF-trap ziv-aflibercept, and ramucirumab, target VEGF signaling[8]. Second-generation antiangiogenic agents, including trebananib, nesvacumab, rebastinib, and MEDI3617, target Ang/Tie2 signaling in clinical development[8,19,24]. However, resistance to these agents, lack of improvement of OS, and common adverse events, including hypertension and bleeding, are challenges faced by current anti-angiogenic therapies. Therefore, combination therapy of VEGF and Ang-2 targeted agents as well as a combination of anti-angiogenic and chemotherapeutic agents, radiation, or immune modulators are being developed accordingly. Recent studies suggested that predicting OS was made possible by periodically measuring serum angiogenic cytokines, especially Ang-2 or VEGF, in patients with HCC treated with sorafenib or lenvatinib [14,25,26]. Moreover, a single-nucleotide polymorphism of Ang-2 is an independent prognostic factor for sorafenib-treated advanced HCC[27]. A high increase in the serum VEGF level after TACE among patients with HCC was associated with distant metastasis and unfavorable outcomes[28,29]. Therefore, identifying tumors using circulating biomarkers that are sensitive to antiangiogenic therapy and predictive of OS and PFS could improve therapeutic approaches.

This study had several limitations. First, this was a single-center study in an HBV-endemic area[30]. Although there was no difference in Ang-2 and VEGF levels according to the etiology of HCC in this study, the results need external validation. Second, the angiogenic biomarkers were measured only once at the time of HCC diagnosis, so the longitudinal profile according to tumor progression or treatment response could not be analyzed. Third, this study did not evaluate the plasma levels of these three angiogenesis factors in normal control subjects or cirrhotic patients because we focused on the role of Ang-1, Ang-2, and VEGF as prognostic rather than diagnostic markers. Fourth, this study did not evaluate other valuable angiogenesis

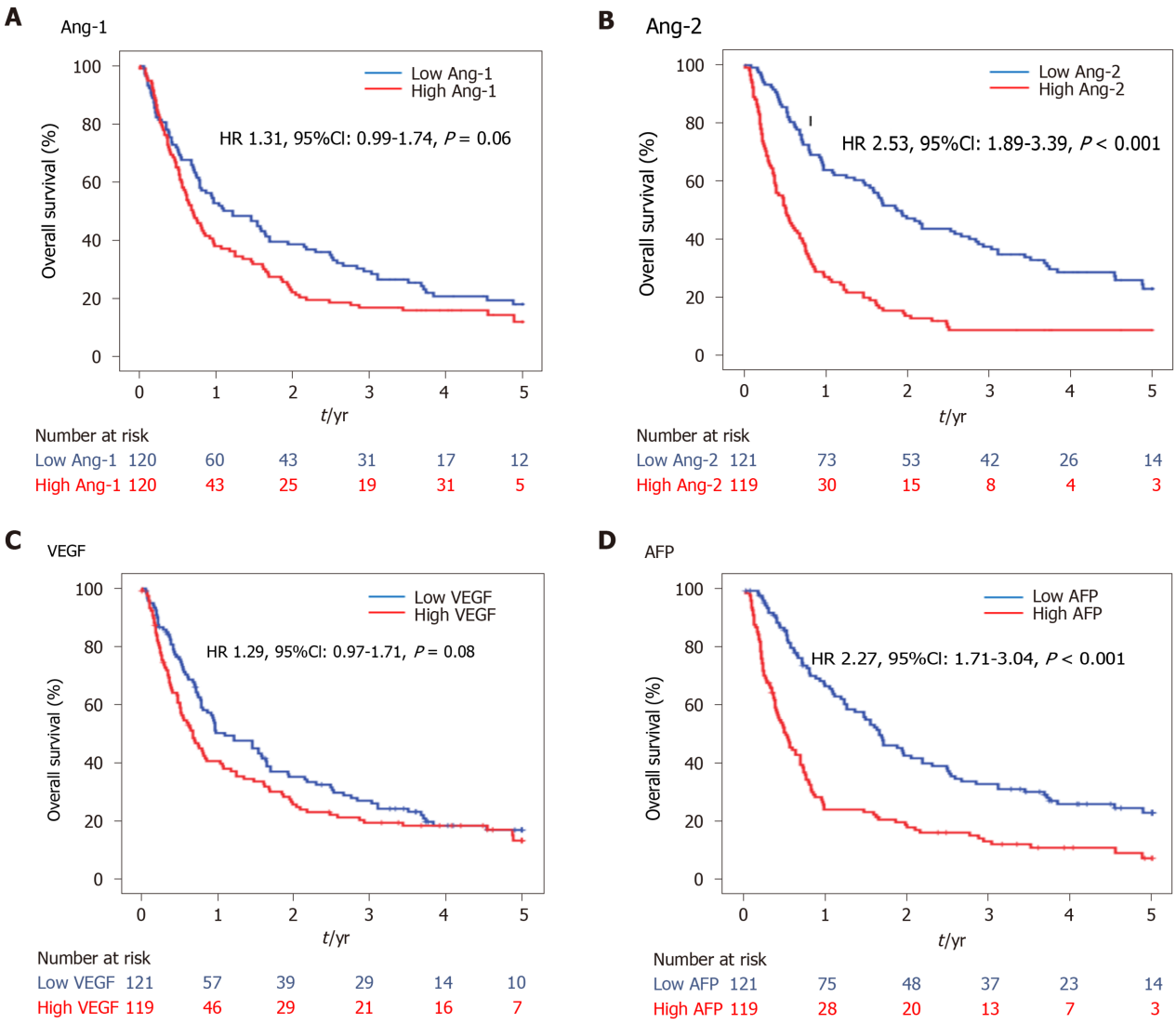


Figure 5 Progression-free survival according to plasma angiogenesis marker level. A: Angiopoietin-1; B: Angiopoietin-2; C: Vascular endothelial growth factor; D: Alpha-fetoprotein. AFP: Alpha-fetoprotein; Ang-1: Angiopoietin-1; Ang-2: Angiopoietin-2; VEGF: Vascular endothelial growth factor; HR: Hazard ratio; CI: Confidence interval.

markers, such as microvessel density, PDGF/PDGFR, FGF/FGFR, and endoglin (CD105)[9,31]. Further comprehensive studies including these angiogenesis markers are hence needed. Lastly, the number of patients who underwent surgery and sorafenib treatment was small. Therefore, it is difficult to generalize that angiogenesis markers can predict the prognosis of patients receiving these treatments.

CONCLUSION

In conclusion, the plasma level of Ang-2 correlated with liver function, tumor stage, and tumor invasiveness. It also performed better than AFP, Ang-1, and VEGF in terms of predicting OS and PFS.

ARTICLE HIGHLIGHTS

Research background

Most hepatocellular carcinomas (HCCs) are hypervascular, with characteristic features of hepatic arterial supply to the tumor. The factors involved in tumor angiogenesis include angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), and vascular endothelial growth factor (VEGF).

Research motivation
Angiogenesis markers can be a potential biomarker.

Research objectives
To investigate the profiles of plasma levels of angiogenesis markers in patients with HCC and evaluate their roles in predicting overall survival (OS) and progression-free survival (PFS).

Research methods
Plasma samples from 240 prospectively enrolled HCC patients in the very early to advanced stages were used to measure the levels of Ang-1, Ang-2, and VEGF. Their associations with clinical characteristics, OS, and PFS were analyzed.

Research results
The plasma level of Ang-2 correlated with liver function, tumor stage, and tumor invasiveness. Multivariable and propensity score-matched analyses revealed Ang-2 Levels with the highest predictive power for OS in patients with HCC.

Research conclusions
The plasma levels of Ang-2 can be a better biomarker than AFP in predicting OS or RFS.

Research perspectives
Identifying HCCs using circulating biomarkers that are sensitive to antiangiogenic therapy and predictive of OS and PFS could improve therapeutic approaches.

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ORIGINAL ARTICLE

Prospective Study

Determinants of disease-specific knowledge among children with inflammatory bowel disease and their parents: A multicentre study

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Abstract

BACKGROUND

Disease knowledge is associated with increased treatment compliance and improvement of symptoms in inflammatory bowel disease (IBD). IBD-knowledge inventory device (IBD-KID) was developed and validated specifically as a tool to measure disease-related knowledge in children with IBD and their parents.

AIM

To prospectively assess the determinants of disease-related knowledge regarding paediatric IBD patients and their parents, using the IBD-KID.

METHODS

A questionnaire-based survey was carried out in paediatric patients and their parents. The determinants of patients' and parents' IBD-KID scores were assessed according to hierarchical linear regression models.

revisions related to important intellectual content of the manuscript; all authors read and approved the final manuscript.

Institutional review board statement: The study was approved by the University Bioethical Committee (Consent No. AKBE/120/16).

Conflict-of-interest statement: The authors declare no conflict of interests.

Data sharing statement: No additional data are available.

CONSORT 2010 statement: The authors have read the CONSORT 2010 Statement, and the manuscript was prepared and revised according to the CONSORT 2010 Statement.

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RESULTS

The study group consisted of 269 IBD patients and 298 parents. The patients' mean (standard deviation, SD) IBD-KID score was 10.87 (± 3.97), while the parents' was 11.95 (± 3.97). Both groups exhibited poor knowledge of the side effects of steroid therapy, the role of surgical treatment in IBD, dietary restrictions and the risks associated with the use of herbal medicines. The patients' IBD-KID scores were statistically associated with patient sex [B coefficient (standard error, SE) = 1.03 (0.44), $P = 0.021$] and patient age [B (SE) = 0.03 (0.01), $P < 0.001$]. The parents' IBD-KID scores were significantly related to patient age [B (SE) = 0.02 (0.01), $P = 0.003$], and treatment with immunosuppressive agent [B (SE) = 1.85 (0.48), $P < 0.001$]. The final models explained 26.9% of the variance of patients' IBD-KID scores and 18.5% of the variance of parents' scores.

CONCLUSION

The variables originating from parents' knowledge were significantly associated with patients' IBD-KID scores. The study results indicate the need to implement better education programmes for patients and parents.

Key Words: Crohn's disease; Inflammatory bowel disease-knowledge inventory device; Knowledge; Parents; Ulcerative colitis

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Core Tip: This was a prospective study that assessed the disease-related knowledge regarding paediatric inflammatory bowel disease (IBD) among children and their parents using previously validated IBD-knowledge inventory device (IBD-KID). The variables originating from parent's knowledge, were significantly associated with patient's IBD-KID score. Parents of patients treated with immunosuppressive agents showed higher diseases-specific knowledge. The results of the study indicate the need to implement better education programmes for patients and parents.

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INTRODUCTION

The burden and prevalence of inflammatory bowel disease (IBD), comprising Crohn's disease (CD) and ulcerative colitis (UC), are increasing among children and adolescents in many regions of the world, including Poland[1-3]. IBD is chronic in nature, potentially occurring with unpredictable periods of exacerbation even during pharmacologically supported remission. The chronic course of the disease is characterised by numerous exacerbations and complications and the need for a wide range of medication (including aminosalicylates (5-ASA), corticosteroids (CS), immunosuppressants, biologics) and, sometimes, surgical treatment, significantly affecting the quality of life of young patients and their families[4]. The disease poses challenges to participation in school activities, social life and travelling, along with dietary restrictions and concerns about the future[5,6]. Parents of sick children and teenagers may experience difficulties understanding and complying with the necessary treatment regimens and dietary recommendations at different stages of the disease. Research has shown that understanding the causes of chronic disease and the principles of treatment results in improved adherence to therapeutic recommendations, facilitated contact with medical personnel and higher quality of life[7-9]. In the case of paediatric patients, especially the youngest ones, the sources of information and the knowledge that parents and guardians gain is of utmost importance. The use of common sense or popular but unproven opinions, instead of reliable sources of

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information, can augment the frustration associated with childhood chronic disease and adversely impact the treatment process. Moreover, the lack of professional dietary or psychological support may further affect the quality of life, resulting in excessive restrictions of a young patient's diet or age-appropriate activity.

IBD-Knowledge Inventory Device (IBD-KID) was developed and validated specifically as a tool to measure disease-related knowledge in children with IBD and their parents[10]. It was previously used among children Canada, Australia and France[10-12]. In Poland, no study has yet assessed the disease-related knowledge of children and teenagers having IBD, nor that of their parents. Therefore, a multicentre study of disease-specific knowledge and its determinants among paediatric patients and their parents was carried out with the use of IBD-KID that was translated into polish and validated.

MATERIALS AND METHODS

Sampling and ethics

The survey involved a convenience sample of patients with IBD and their parents from four Polish university centres specialising in paediatric gastroenterology between 2016 and 2018. The participating departments were based in Warsaw, Olsztyn, Wroclaw and Krakow. Patients aged 10 years and older and their parents or guardians completed the questionnaire used in the survey. In the case of younger patients, parents filled out the questionnaires.

The study was approved by the University Bioethical Committee (Consent No AKBE/120/16). After participants were informed about the study aims, only those who provided their consent were invited to fill the questionnaire. Each pair of surveys (or survey) was assigned a unique code to preserve respondents' anonymity.

Questionnaire

The questionnaire used in the survey consisted of the IBD-KID instrument that is available at https://cdn-links.lww.com/permalink/mpg/a/mpg_2013_10_11_otley_135_sdc1.pdf; the 23 items asking about general knowledge (2), IBD course (6), risk factors (4), therapeutic modes and adverse effects of treatment (11). Scoring of the questionnaire was one point for each correct answer with no negative marking; the maximum score was 23 points. Demographic characteristics of patients and parents and membership of the Polish patient Crohn's & Ulcerative Colitis Association *J-elita* were also collected. Parents were also queried about preferred sources of information and asked to provide a self-assessment of their knowledge about their child's disease.

The Polish version of the BID-KID scale used in the study was obtained after cultural adaptation consisting of three stages, including forward translation, backward translation and cognitive debriefing, according to the guidelines provided by Prof. Anthony Otley, the author of the original version of the scale[10]. The conformance to the guidelines provided by the authors of the scale was confirmed with the validation certificate from 31 March 2015[13].

Statistical analysis

Statistical analysis was carried out using IBM SPSS v25 software (IBM Corp. Armonk, NY, United States). The mean and standard deviation were provided for the continuous variables as well as absolute and relative frequencies for categorical variables. The Kolmogorov–Smirnoff test was applied to check whether continuous variables conformed to the normal distribution. The internal consistency of the IBD-KID scale was assessed using Cronbach's alpha. A P value < 0.05 was deemed to be statistically significant.

In the first stage of analysis, the association between IBD-KID scores and potential predictors was assessed using univariate linear regression. Next, hierarchical linear regression models were developed for both patients' and parents' IBD-KID scores. In multivariate models, only those independent variables for which the p-value in the univariate model was at least 0.1 were included.

Hierarchical multivariate linear regression modelling was performed for both parents' and patients' IBD-KID scores. In three consecutive steps of the hierarchical multivariate regression for patients' scores, the following independent variables were introduced: (1) Patient's demographic characteristics and self-assessment of knowledge; (2) Parent's IBD-KID score and their self-assessment of knowledge, membership in the J-elita Association and the use of the Internet as a source of knowledge; and (3) The use of treatment options.

Hierarchical regression modelling for parents' IBD-KID scores also consisted of three steps, in which the following variables were introduced: (1) Patient's demographic characteristics and their self-assessment of knowledge; (2) Parent's demographic characteristics, self-assessment of knowledge, membership in the J-elita Association and the use of books and journals as sources of knowledge; and (3) The use of treatment options.

Hierarchical regression was chosen to expose the relative importance of variables potentially associated with the level of IBD-KID scores in both groups of respondents. The assumption was also made that a parent's IBD-KID score might play the role of a predictor of the patient's score.

Unstandardised regression coefficients (B), standard errors (SE), standardised regression coefficients (beta), 95% confidence intervals, and *P*-values were reported for independent variables included in the univariate analysis. For those included in the hierarchical linear regression models, 95% confidence intervals were omitted. In the case of hierarchical linear regression models, standardised R2 values and F for the change in R2 were also provided.

RESULTS

Study group characteristics

The survey was completed by 269 IBD patient-parent pairs (for patients 10 years and older) and 29 parents of patients under 10 years old. The mean age (SD) of patients was 167.3 (37.5) mo, and the mean duration of disease (SD) was 40.7 (33.8) mo. Table 1 displays patient characteristics.

IBD-KID scores

Cronbach's alpha coefficient for patients' IBD-KID scale was 0.75, while this value for parents was 0.60. The patients' and parents' mean (SD) IBD-KID scores were 10.87 (3.97) and 11.95 (3.97), respectively. Both patients with CD and their parents reached higher scores [mean (SD): 11.06 (3.99) and 12.18 (4.14), respectively] than those with UC and their parents [10.52 (SD ± 3.93) and 11.52 (SD ± 3.60), respectively]. The percentage of correct answers given by patients was in the range of 74.83% (item No 13) and 12.08% (item No 20). In the case of parents' responses, the range was 87.92% (item No 3) - 11.74 (item No 17). The distribution of correct answers to the KID-IBD scale items is shown in the supplementary file (Supplementary Table 1).

Regression modelling of IBD-KID scores

Univariate linear regression analysis showed that patients' scores were significantly associated with patient age and sex, patient's and parent's self-rated knowledge, membership in the Jelita Association, parent's use of the Internet as a source of knowledge and parent's IBD-KID score (Table 2, Supplementary Figure 1). Furthermore, nutritional and surgical therapy and the use of immunosuppressive or biological agents were also statistically related to the patient's score. Parents' IBD-KID scores showed a significant relationship with parent sex and education, place of residence, parent's and patient's self-assessment of knowledge, membership in the J-elita Association, and the use of books or journals for accessing knowledge about the disease (Table 2, Supplementary Figure 2). As in the case of the patients' scores, the use of immunosuppressive or biological agents was associated with higher scores.

Tables 3 and 4 show the final model obtained with hierarchical linear regression for the IBD-KID scores of both patients and parents, respectively (details of all three models are shown in Supplementary Tables 2 and 3). The assumptions for multiple linear regression were met for both models. In the first step, patient sex and age, place of residence and self-assessment of knowledge were introduced to the model for the patient's IBD-KID score. All four independent variables were significant predictors, as the resulting model explained 14.1% of the variance of the score (Supplementary Table 2). In the second step, parent's self-assessment of knowledge, J-elita Association membership, using the Internet as a source of knowledge and parent's IBD-KID score were added. The resulting model explained an additional 12.7% of the variance of the patient's IBD-KID score (Supplementary Table 2). The third step included variables reflecting specific therapeutic modalities used in a patient. This technique explained an additional 0.1% of the variance, and the change in R^2 was not statistically significant (Table 3).

Table 1 Demographics of inflammatory bowel disease patients participating in inflammatory bowel disease-knowledge inventory device study

Variable	Categories of variable	Patients, *n* (%)
Patient's gender	Female	140 (52.0)
	Male	129 (48.0)
Parent's gender	Female	232 (80.0)
	Male	58 (20.0)
Place of residence	Rural	172 (57.9)
	Urban	125 (41.1)
Parent's education	Lower than secondary	48 (16.2)
	Secondary	123 (41.6)
	Higher than secondary	125 (42.2)
Disease type	CD[1]	196 (65.8)
	UC[2]	102 (34.2)
IBD family history		50 (16.8)
J-elita membership		64 (21.8)
Treatment modes		
Aminosalicylates	All patients	248 (83.2)
	CD	160 (81.6)
	UC	88 (86.3)
Immunomodulators	All patients	205 (68.8)
	CD	143 (73.0)
	UC	62 (60.8)
Biologic therapy	All patients	99 (33.2)
	CD	81 (41.3)
	UC	18 (17.6)
Exclusive enteral nutrition	All patients	98 (32.9)
	CD	85 (43.4)
	UC	13 (12.7)
Dietary counselling	All patients	90 (30.2)
	CD	67 (34.2)
	UC	23 (22.5)
Psychological support	All patients	53 (17.8)
	CD	34 (17.3)
	UC	19 (18.6)
Surgical treatment	All patients	30 (10.1)
	CD	27 (13.8)
	UC	3 (2.9)
Patient's self-assessment of knowledge	Good	120 (45.1)
	Average	125 (47.0)
	Low	21 (7.9)
Parent's self-assessment of knowledge	Good	152 (51.7)
	Average	122 (41.5)

Low	20 (6.8)
Sources of parent's information about disease	
Physician	241 (80.9)
Nurse	66 (22.1)
Internet	197 (66.1)
Journals	82 (27.5)
Books	77 (25.8)

[1]One hundred and seventy-four questionnaires filled both by a child and a parent and 22 only by parents.
[2]Nity-five questionnaires filled both by a child and a parent and 7 filled only by parents. CD: Crohn's disease; UC: Ulcerative colitis.

As for the parents' IBD-KID scores, the first step of hierarchical linear regression introduced patient age, patient's self-assessment of knowledge and place of residence. All three variables exerted a significant effect on the parent's IBD-KID score (Supplementary Table 3). The model explained 7.5% of the variance of the parent's score. The second step added the parental education level and self-assessment of knowledge, J-elita Association membership and the use of books and journal as sources of knowledge (Supplementary Table 3). The change in R^2 with this model was equal to 0.045 and statistically significant. The third step introduced the variables reflecting treatment modalities. The results explained a further 6.5% (18.5% in total) of the variance of the parent's score. In the final model, patient age, parental education level, and the use of immunosuppressive agent were significant predictors of the parent's score (Table 4).

DISCUSSION

This study assessed the disease-specific knowledge of a large sample of paediatric IBD patients and their parents. This investigation is the first to be based on the dedicated tool in this particular group in Poland. We found that both patients and their parents had insufficient IBD-related knowledge. Univariate models developed for the patients' IBD-KID scores showed that these scores were higher among older than younger patients, among girls compared to boys, among patients who more highly assessed their knowledge about the disease and whose parents did the same. Furthermore, higher scores were found among patients whose parents indicated the Internet as a main source of information, reported membership in the J-elita Association and noted nutritional, surgical immunosuppressive or biological treatment. The patient's score was also positively associated with the parent's score. A fully developed hierarchical regression model maintained a significant association between patients' scores and their gender and age, parents' self-rated knowledge, and IBD-KID scores. Univariate analysis revealed that parents' scores were significantly higher among women compared to men, among the parents of younger *vs* older children, among those with higher self-rated knowledge and whose children better assessed their knowledge, among parents with higher *vs* lower levels of education, and among those using books and journals as sources of information. Membership in a patients' association and earlier immunosuppressive or biological treatment in a child were also associated with a higher score. The hierarchical regression model showed that a significant association was retained only for patient's age, prior immunosuppressive therapy and parental education level.

The average scores of our study's parents and patients agree with results obtained in paediatric studies carried out using the same questionnaire in Canada, Australia and New Zealand[11,14]. Other studies reported similar findings on factors influencing the IBD-KID scores as in our study. For example, a significant relationship between patients' and parents' IBD-KID scores and self-rated knowledge was reported by Haaland *et al*[10]. We have shown, as have they, a correlation between parents' level of education and parents' but not patients' scores [10]. However, this relationship was not observed by Vernon-Roberts *et al*[15] in an investigation using a modified version of the questionnaire: IBD-KID2.

We have found no correlation between IBD-KID score and disease duration. On the other hand, both uni- and multivariate analysis revealed a link between the IBD-KID

Table 2 Univariate linear regression models for patient's and parent's inflammatory bowel disease-knowledge inventory device scores

Independent variables	Categories of independent variables	Patient's score			
		B (SE)	95%CI		*P* value
Univariate models for patient's IBD-KID score					
Patient's age		0.03 (0.01)	0.02 to 0.05	0.24	< 0.001
Parent's sex		-0.69 (0.61)	-1.89 to 0.52	-0.07	0.26
Patient's sex		-1.12 (0.48)	-2.07 to -0.17	-0.14	0.021
Place of residence		0.86 (0.48)	-0.09 to 1.82	0.11	0.076
Type of disease	UC *vs* CD	-0.54 (0.51)	-1.54 to 0.46	-0.07	0.29
Time from the diagnosis		0.01 (0.01)	-0.01 to 0.02	0.05	0.43
Parent's self-assessment	Low *vs* medium	-2.29 (0.94)	-4.15 to -0.43	-0.15	0.016
	Good *vs* medium	1.63 (0.49)	0.67 to 2.59	0.21	0.001
Patient's self-assessment	Low *vs* medium	-3.25 (0.90)	-5.03 to -1.48	-0.22	< 0.001
	Good *vs* medium	0.90 (0.49)	-0.06 to 1.86	0.11	0.067
Parents level of education	Lower than sec. *vs* sec.	0.23 (0.70)	-1.15 to 1.62	0.02	0.74
	Higher than sec. *vs* sec.	0.61 (0.53)	-0.43 to 1.66	0.08	0.25
Source: Physician	Yes *vs* no	-0.10 (0.63)	-1.34 to 1.14	-0.01	0.87
Source: Nurse	Yes *vs* no	-0.10 (0.59)	-1.26 to 1.05	-0.01	0.86
Source: Books	Yes *vs* no	0.61 (0.57)	-0.51 to 1.73	0.07	0.28
Source: Journals	Yes *vs* no	0.77 (0.54)	-0.30 to 1.84	0.09	0.15
Source: Internet	Yes *vs* no	1.22 (0.50)	0.23 to 2.21	0.15	0.016
J-elita Ass. Membership	Yes *vs* no	1.22 (0.59)	0.06 to 2.37	0.13	0.039
Close family	Yes *vs* no	1.16 (0.85)	-0.51 to 2.83	0.08	0.17
Family 2nd grade	Yes *vs* no	0.43 (0.85)	-1.24 to 2.11	0.03	0.61
Parent's score	Yes *vs* no	0.42 (0.06)	0.31 to 0.53	0.41	< 0.001
Nutritional treatment	Yes *vs* no	1.03 (0.51)	0.01 to 2.04	0.12	0.047
Psychologist	Yes *vs* no	0.73 (0.63)	-0.51 to 1.98	0.07	0.25
Dietitian	Yes *vs* no	-0.12 (0.53)	-1.15 to 0.92	-0.01	0.82
Surgical treatment	Yes *vs* no	2.36 (0.80)	0.79 to 3.93	0.18	0.003
Salicylates	Yes *vs* no	0.19 (0.66)	-1.12 to 1.49	0.02	0.78
Immunosuppressive agent	Yes *vs* no	1.15 (0.52)	0.14 to 2.17	0.14	0.026
Biological agent	Yes *vs* no	1.29 (0.52)	0.28 to 2.31	0.15	0.013
Univariate models for parent's IBD-KID score					
Patient's age		-0.01 (0.01)	-0.02 to 0	-0.11	0.073
Parent's sex		-1.23 (0.54)	-2.3 to -0.15	-0.13	0.025
Patient's sex		0.23 (0.48)	-0.73 to 1.18	0.03	0.64
Place of residence		1.45 (0.48)	0.51 to 2.39	0.18	0.003
Type of disease	UC *vs* CD	-0.66 (0.49)	-1.61 to 0.30	-0.08	0.18
Time from the diagnosis		0.01 (0.01)	-0.01 to 0.02	0.06	0.32
Parent's self-assessment	Low *vs* medium	-2.97 (0.92)	-4.78 to -1.16	-0.19	0.001
	Good *vs* medium	1.15 (0.46)	0.24 to 2.07	0.15	0.014

Patient's self-assessment	Low *vs* medium	-3.64 (0.89)	-5.39 to -1.88	-0.25	0.000
	Good *vs* medium	0.53 (0.48)	-0.43 to 1.48	0.07	0.28
Parents level of education	Lower than sec. *vs* sec.	-0.89 (0.67)	-2.21 to 0.43	-0.08	0.18
	Higher than sec. *vs* sec.	1.17 (0.50)	0.20 to 2.15	0.15	0.019
Source: physician	Yes *vs* no	-0.05 (0.59)	-1.20 to 1.10	0.00	0.93
Source: nurse	Yes *vs* no	-0.79 (0.55)	-1.88 to 0.30	-0.08	0.16
Source: books	Yes *vs* no	1.28 (0.52)	0.24 to 2.31	0.14	0.016
Source: journals	Yes *vs* no	1.31 (0.51)	0.30 to 2.32	0.15	0.011
Source: Internet	Yes *vs* no	0.67 (0.49)	-0.28 to 1.63	0.08	0.17
J-elita Ass. Membership	Yes *vs* no	1.45 (0.55)	0.36 to 2.53	0.15	0.009
Close family	Yes *vs* no	0.30 (0.81)	-1.29 to 1.90	0.02	0.71
Family 2nd grade	Yes *vs* no	0.87 (0.84)	-0.78 to 2.52	0.06	0.30
Nutritional treatment	Yes *vs* no	0.97 (0.49)	0 to 1.93	0.11	0.050
Psychologist	Yes *vs* no	0.90 (0.60)	-0.28 to 2.08	0.09	0.14
Dietitian	Yes *vs* no	0.19 (0.50)	-0.81 to 1.18	0.02	0.71
Surgical treatment	Yes *vs* no	1.27 (0.76)	-0.23 to 2.77	0.10	0.097
Salicylates	Yes *vs* no	0.50 (0.62)	-0.71 to 1.72	0.05	0.41
Immunosuppressive agent	Yes *vs* no	1.45 (0.49)	0.48 to 2.41	0.17	0.003
Biological agent	Yes *vs* no	1.23 (0.49)	0.27 to 2.18	0.15	0.012

B: Unstandardised regression coefficient; SE: Standard error; : Standardised regression coefficient; 95%CI: 95% confidentiality interval: sec.: Secondary education; IBD-KID: Inflammatory bowel disease-knowledge inventory device.

scores and patient's age. Other authors have suggested that the patients' and parents' core knowledge is obtained during the period of diagnosis of the disease. However, while patients' awareness and knowledge of the disease increases with age, parents gradually become less involved, eventually forgetting some information[10,16].

Numerous studies, both in children and adults with IBD, confirm that membership in organisations supporting IBD patients increases disease awareness and disease-specific knowledge[14,17,18]. Our study findings also seem to confirm these observations. Membership in the patient Crohn's and Ulcerative Colitis Association J-elita correlated with higher patient and parent scores. J-elita is a Polish patient association of adults and children diagnosed with IBD and a member of The European Federation of Crohn's & Ulcerative Colitis Associations. The aims of J-elita are to raise awareness about IBD and improve the life of the several thousand people living with IBD in Poland. This outcome may reflect patients' and their parents' involvement in the organisation's educational activities. Interestingly, the effect of membership was not retained in the multivariate models, possibly because both membership and higher scores could be achieved by more active patients or parents as reflected by the preserved effect of self-rated knowledge.

Our study revealed that the therapy modes used in a patient might be related to both patients' and parents' scores. Concerning exclusive enteral nutrition, which is recommended as a treatment inducing remission in paediatric CD patients[19], the specific nature of this treatment and the need for medical follow-up may explain why these patients usually remain longer under close medical monitoring during the first period of illness. Consequently, they have more frequent contact with qualified staff who can provide them with reliable information. Presumably, this situation contributes to greater and more systematic involvement in the treatment process.

In the case of the parent's score, a significant association was present for immunosuppressive treatment even after adjusting for other factors included in the multivariate model. Both immunosuppressive therapy and biological treatment are applied in more severe forms of IBD, and surgical intervention may be necessary when treating complications. Such patients usually have a longer history of multiple hospitalisations and treatment of exacerbations. Studies performed in adult IBD patients showed that more frequent hospitalisations were associated with better disease-

Table 3 Hierarchical linear regression for patient's inflammatory bowel disease-knowledge inventory device score as an independent variables (model 3; details of model 1 and 2 are shown in Supplementary Table 2)

Independent variables		Model 3		
		B (SE)	Beta	*P* value
Patient's sex		-1.03 (0.44)	-0.13	0.021
Place of residence		0.41 (0.45)	0.05	0.37
Patient's age		0.03 (0.01)	0.24	< 0.001
Patient's self-assessment of knowledge	Low *vs* medium	-1.87 (1.46)	-0.12	0.20
	Good *vs* medium	-0.74 (0.74)	-0.09	0.32
Parent's self-assessment of knowledge	Low *vs* medium	-1.24 (1.68)	-0.08	0.46
	Good *vs* medium	-1.66 (0.74)	-0.21	0.026
J-elita membership		0.69 (0.55)	0.07	0.21
Internet as a source of knowledge		0.67 (0.47)	0.08	0.16
Parent's IBD-KID score		0.33 (0.06)	0.32	< 0.001
Immunosuppressive agent		-0.12 (0.51)	-0.01	0.82
Biological agent		0.26 (0.52)	0.03	0.61
Nutritional therapy		0.38 (0.48)	0.05	0.43
Surgical therapy		1.26 (0.76)	0.10	0.098
Raw/Corrected R^2		0.311/0.269		
F for change in R^2		1.108		
P value for R^2 change		0.35		

P value for the significance of the independent variable in the multivariate model. Dependent variable: patient's IBD-KID score, model assessment: model 3 – df = 14, F = 7.562, *P* < 0.001. IBD-KID: Inflammatory bowel disease-knowledge inventory device; B: Unstandardised regression coefficient; SE: Standard error; Beta: Standardised regression coefficient; sec.: Secondary education.

specific knowledge on the part of patients[20]. Therefore, in such circumstances, paediatric patients and their parents are also highly likely to be strongly motivated and more engaged in searching for additional information about the disease. Moreover, it is probable that greater parent's knowledge may lead to better disease management; the immunosuppressive therapy is currently most recommended in IBD.

In general, our study revealed that parents were much more likely to give correct answers compared to children (Supplementary material, Table 1). Both patients and parents revealed relatively adequate knowledge about anatomical conditions, risk of disease transmission, factors leading to exacerbations, and principles of treatment in remission. Areas of insufficient knowledge included the side effects of steroid therapy, the role of surgical treatment in IBD, dietary restrictions and the risks associated with the use of herbal medicines.

The pattern of knowledge seems very similar to that found in other countries[10-12, 14]. Fewer than 40% of children and slightly more than 50% of parents were aware that the disease itself, as well as poor nutrition and the use of steroids, could cause osteoporosis. Fewer than one-third of respondents knew that steroids could suppress a child's growth. Lack of sufficient knowledge regarding side effects caused by corticosteroids may be at least partially related to actual recommendations of avoiding steroid therapy, particularly on a long-term basis. Therefore, the side effects of steroid therapy may currently be seen less often than decades ago.

Particularly important is the lack of proper knowledge of nutrition for sick children. A significant proportion of those surveyed expressed the opinion that dietary restrictions, including the withdrawal of certain products from the diet, could prevent aggravation of the disease. This misunderstanding is a cause for serious concern, as frequent and unjustified food avoidance may influence the nutritional status and general well-being of paediatric patients[6]. Lack of awareness of the risks associated with the use of herbal medicines related to their potential interaction with other drugs turned out to be another area of poor knowledge. Similar results were obtained using

Table 4 Hierarchical linear regression for the parent's inflammatory bowel disease-knowledge inventory device score as independent variables (model 3; details of model 1 and 2 are shown in Supplementary Table 3)

Independent variables		Model 3		
		B (SE)	Beta	*P* value
Place of residence		0.77 (0.44)	0.11	0.085
Patient's age		-0.02 (0.01)	-0.18	0.003
Patient's self-assessment of knowledge	Low *vs* medium	0.36 (1.56)	0.02	0.89
	Good *vs* medium	-1.06 (0.72)	-0.15	0.14
Parent's self-assessment of knowledge	Low *vs* medium	-2.65 (1.73)	-0.17	0.13
	Good *vs* medium	-1.10 (0.71)	-0.15	0.12
J-elita membership		-0.02 (0.56)	0.00	0.97
Parent's sex		-0.54 (0.54)	-0.06	0.32
Source books		0.66 (0.55)	0.08	0.23
Source journals		0.38 (0.55)	0.05	0.49
Parent's level of education	Lower than sec. *vs* sec.	-1.99 (0.63)	-0.21	0.002
	Higher than sec. *vs* sec.	-1.14 (0.48)	-0.16	0.019
Immunosuppressive agent		1.85 (0.48)	0.24	< 0.001
Biological agent		0.33 (0.52)	0.04	0.53
Nutritional therapy		0.58 (0.47)	0.08	0.23
Surgical therapy		1.17 (0.73)	0.10	0.11
Raw/Corrected R^2		0.238/0.185		
F for change in R^2		5.550		
P value for R^2 change		< 0.001		

P value for the significance of the independent variable in the multivariate model. Dependent variable: Parent's inflammatory bowel disease-knowledge inventory device score. Model assessment: Model 3 – df = 16, F = 4.440, $P < 0.001$. B: Unstandardised regression coefficient; SE: Standard error; Beta: Standardised regression coefficient; sec.: Secondary education.

the shortened, simplified research tool IBD-KID2[21]. Therefore, this problem is not an isolated concern affecting only Polish patients. We can assume that certain information that doctors and medical staff consider obvious is not properly passed on to patients and their parents.

Proper education of patients with IBD, including paediatric patients and their parents, regarding their disease and treatment principles, may have a significant impact on the treatment compliance and ability to self-cope with the disease[22-24]. Research has also proved that the benefits of educational programmes outweigh anxiety regarding greater disease knowledge[25].

Better knowledge regarding drug mechanisms and drug side effects as well as better IBD-specific knowledge have also been shown to contribute to treatment compliance[7,8,26,27].

The strength of our study is its sample size; to date, this study is the largest investigation that has assessed disease-related knowledge in children with IBD. Polish validation of the questionnaire allowed comparing the IBD knowledge of both children with this disease and their parents to their peers in different countries undergoing different types of health care. The survey's main limitations include the use of the first version of the IBD-KID, which was recently revised. The new, simplified and shortened version, the IBD-KID2, was not available at the time of our study.

CONCLUSION

This study identified gaps in the IBD-related knowledge of Polish children with IBD

and their parents. The variables originating from the parents' knowledge, apart from the patients' demographic characteristics, were significantly associated with the patients' IBD-KID scores. Parents of patients treated with immunosuppressive agents showed higher disease-specific knowledge. The study results also confirmed that information acquired from a patient organisation can play a vital role in correct IBD-related knowledge. This finding forms a basis for intensified action aimed at creating educational programmes. Increased awareness of the disease and knowledge about treatment can have a positive effect on compliance with therapeutic recommendations.

ARTICLE HIGHLIGHTS

Research background

Patient knowledge is associated with increased treatment compliance and improvement of symptoms in a variety of chronic diseases, including inflammatory bowel disease (IBD). IBD-knowledge inventory device (IBD-KID) was developed and validated specifically as a tool to measure disease-related knowledge in children with IBD and their parents.

Research motivation

Until now, assessment of IBD-related knowledge in children with IBD and their parents were performed in Canada, Australia and France. However, the results of these studies cannot be simply projected to Poland due to the differences in lifestyle, diet and healthcare model. The importance of such factors for understanding and knowledge about disease in Polish patients with IBD requires a separate study.

Research objectives

Our present study aimed to assess disease-related knowledge in children with IBD and their parents.

Research methods

A questionnaire-based survey was carried out in 269 children with IBD and 298 parents. The determinants of patients' and parents' IBD-KID scores were assessed with hierarchical linear regression models.

Research results

We found that both patients with IBD and their parents had insufficient IBD-related knowledge. Patients' IBD-KID scores were higher among older than younger patients, among girls compared to boys, among patients who more highly assessed their knowledge about the disease and whose parents did the same. Furthermore, higher scores were found among patients whose parents indicated the Internet as a main source of information, reported membership in the patient Crohn's and Ulcerative Colitis Association J-elita and noted nutritional, surgical immunosuppressive or biological treatment. The patient's score was also positively associated with the parent's score.

Research conclusions

This study identified gaps in the disease-related knowledge of Polish children with IBD and their parents. Increased awareness of the disease and knowledge about treatment can have a positive effect on compliance with therapeutic recommendations.

Research perspectives

Further studies on disease-related knowledge among patients with IBD in countries undergoing economic transformation are needed. IBD-KID may be a good tool to assess transition of adolescents with IBD from pediatric to adult care. It could be also used in assessment of any intervention aimed to increase the level of patients' self-care.

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LETTER TO THE EDITOR

Is there higher percentage of undetected osteopenia and osteoporosis among patients with ulcerative colitis in Saudi Arabia?

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Abstract

Detection of prevalence and development of osteopenia or osteoporosis in patients with inflammatory bowel disease using only bone mineral density could be inappropriate to detect all individuals at risk for osteoporosis. Numerous patients could remain undetected by using only bone mineral density as a screening method, especially in patients with ulcerative colitis. Therefore, trabecular bone score should be used as a complementary method.

Key Words: Inflammatory bowel disease; Osteoporosis; Bone mineral density; Trabecular bone score

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Core Tip: Lower body mass index is a predictor of osteoporosis in patients with inflammatory bowel disease however, fracture risk can be increased desite normal bone mineral density. Therefore, use of trabecular bone score would ensure recruitment of individuals with normal bone mineral density at increased risk of fractures in trials.

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TO THE EDITOR

We read with a great interest the study by Ewid *et al*[1], that evaluated the connection between inflammatory bowel disease (IBD) and bone mineral density (BMD) in a sample of adult Saudi patients with IBD from a single center.

One important finding of this study was that Crohn's disease (CD) patients are at a higher risk of developing both osteoporosis and osteopenia than ulcerative colitis (UC) patients, even though the study included more patients with severe clinical disease activity and endoscopic activity in the UC group than in the CD group (25% *vs* 5%, respectively). In spite of disease severity, 78% of UC patients had normal BMD *vs* 44% of CD patients. Fracture risk can be increased despite normal BMD. Maldonado *et al*[2] suggested that current screening does not include all IBD patients at increased risk of fractures. We propose using trabecular bone score (TBS) as a complementary tool; as it discriminates patients at higher risk of fractures better than BMD[3].

According to demographic characteristics in the study, mesalamine use was significantly higher in the UC compared to CD group (81% *vs* 10%, respectively). Due to the sample size of UC group an analysis of BMD according to mesalamine use probably could not have been performed nevertheless, the analysis would be highly informative. Hence, we postulate a question: could mesalamine use have contributed to lower risk of developing osteoporosis in UC compared to the CD cohort?

Krajcovicova *et al*[4] observed no significant difference in the prevalence of low BMD in groups of patients with UC and CD. They observed that corticosteroid therapy and menopause had significant negative effects, whereas combined treatment with an anti-tumor necrosis factor (TNF) α agent and azathioprine had a significant positive effect on Δ BMD at the lumbar spine (BMDL) per year[4].

The study also showed that low BMI is a risk factor for reduced BMD. According to Fawzy *et al*[5] and Steinschneider *et al*[6], increased BMD commonly reported in overweight women may be a result of soft tissue interference, further supporting addition of TBS as a screening method for osteoporosis in research and clinical practice.

An unexpected finding in this study is the lack of correlation between steroid use and BMD. Paggiosi *et al*[7] demonstrated that TBS alone and BMDL in combination with TBS, but not BMDL alone were able to discriminate between glucocorticoid-treated and glucocorticoid-naïve women. In the study by Gulyás *et al*[8] TNF-α inhibition slowed down generalized bone loss in rheumatoid arthritis and ankylosing spondylitis. Effect of anti TNF therapy on bone metabolism was not confirmed by Ewid *et al*[1] hence, the effect is questionable in IBD.

Addition of TBS would enable inclusion of individuals with normal BMD at increased fracture risk. In conclusion, by including both screening methods osteoporotic fracture risk in UC *vs* CD cohort could be assessed more accurately.

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ABOUT COVER

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EDITORIAL

Asymptomatic small intestinal ulcerative lesions: Obesity and *Helicobacter pylori* are likely to be risk factors

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Abstract

It is often difficult to explain why ulcerative lesions are found in the small intestine because there are no obvious aggressors such as gastric acid. In particular, the treatment of small intestinal ulcerative lesions in asymptomatic patients with no symptoms, normal physical examinations, and normal blood test findings is not well documented. According to a summary of capsule endoscopy studies in healthy subjects, approximately 10% of subjects have small intestinal mucosal breaks. The number of mucosal breaks in these instances is approximately 1-3. We examined small intestinal mucosal breaks in healthy subjects recruited from our past two studies. Mucosal breaks were observed in approximately 10% of subjects, and the average number was 0.24 ± 1.21. The number of mucosal breaks in the small intestine was correlated with body mass index and was significantly higher in *Helicobacter pylori*-infected subjects and higher in males. These results indicate that 1-2 small ulcerative lesions, such as erosions in the small intestine, can be considered to be in the normal range, and close examination is not required. It is assumed that a follow-up medical examination is required for such asymptomatic persons. The presence of many small ulcerative lesions or an unequivocal ulcer indicates an abnormality for which close examination is desired. However, in many cases, it is sufficient to scrutinize after detecting anemia, but it is difficult to make a judgment due to insufficient reports, and future studies are required.

Key Words: Small intestine; Mucosal break; Ulcer; Asymptomatic; Obesity; *Helicobacter pylori*

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Core Tip: Approximately 10% of asymptomatic subjects with normal blood tests have

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small intestinal mucosal breaks. A reanalysis of our previous studies showed that small intestinal mucosal breaks were strongly correlated with body mass index, were significantly more prevalent in *Helicobacter pylori*-infected individuals and were more prevalent in men. These may be risk factors for small intestinal mucosal breaks. Overall, 1-2 small ulcerative lesions in asymptomatic patients are considered to be in the normal range and do not require close examination, and it is assumed that a follow-up medical examination is required.

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INTRODUCTION

Ulcerative lesions are less common in the small intestine, except in the duodenum, than in the stomach. Even if some small ulcerative lesions such as erosions are found in the stomach, we do not care much because of the many accumulated reports and experiences. However, we are concerned with the cause of ulcerative lesions of the small intestine. This is because the small intestine lacks obvious aggressors such as gastric acid, making it difficult to explain the cause of ulcerative lesions. Moreover, there is insufficient knowledge about ulcerative lesions of the small intestine.

Screening colonoscopy occasionally detects a small number of small ulcerative lesions at the terminal ileum. Patients with small ulcerative lesions at the terminal ileum who do not take nonsteroidal anti-inflammatory drugs (NSAIDs) are often doubtful about Crohn's disease (CD) in endoscopic results reports. Some patients had small ulcerative lesions that could potentially progress to CD, and a case was reported [1]. Recently, a report described that CD was detected in 0.28% (13 out of 4640 cases) by colorectal cancer screening[2]. The report indicated that ileal ulcers were detected in 6 of 13 cases, suggesting that ileal ulcers were associated with CD. Four of the 6 patients had no lesions other than ileal ulcers, and the simple endoscopic score for CD was 2-6 points. In other words, some patients had a slightly larger ileal ulcer; however, all patients were scheduled only for a followed up without treatment. It is considered difficult to make an endoscopic diagnosis of the 4 patients with CD, and it is not stated whether granuloma was detected by histological examination. The diagnosis of CD is difficult in the early stages, but the patients in this study did not appear to meet the diagnostic criteria for CD[3]. The question remains as to the diagnosis of CD in these patients, and the follow-up results are considered important.

Subjects who were asymptomatic with no abnormalities in blood tests or physical tests and were not taking medication were defined as healthy subjects. This may include patients with asymptomatic diseases; however, these were subjects without any obvious disease. It is not easy to frequently follow-up with an endoscopy for asymptomatic small ulcerative lesions of the small intestine identified the screening test of healthy subjects because of the physical and financial burdens imposed on the subject. In addition, even if an asymptomatic small ulcerative lesion is found at the terminal ileum, it is considered to be rare and not enough reason to conduct close examination, such as capsule endoscopy. However, it is not clear whether this research policy is correct. This is because the treatment of small intestinal ulcerative lesions in asymptomatic patients is not reported. This is thought to be because reports on the proportion of ulcerative lesions in the small intestine found in healthy subjects and the cause of small intestinal ulcerative lesions are extremely few. In this paper, we summarize how many small intestinal ulcerative lesions are found in healthy subjects, analyze the degree of appearance of small intestinal ulcerative lesions, and investigate the causes of small intestinal ulcerative lesions where possible. Then, we would like to consider the research policy when small intestinal ulcerative lesions are found.

ANALYSIS OF SMALL INTESTINAL ULCERATIVE LESIONS IN HEALTHY SUBJECTS

Definition of small intestinal ulcerative lesions

Ulcerative lesions of the small intestine are often evaluated as mucosal breaks. A mucosal break is a lesion in which white slough is observed, and the degree varies from small ulcerative lesion erosion (Figure 1A) to ulcer (Figure 1B). It is difficult to clearly distinguish this lesion, and there are many cases in which it is not clear which category a specific lesion should be classified into, as shown in Figure 1C. This ulcerative lesion has white slough over 3 mm and approximately 5 mm. Since it is expected that the judgments by researchers will be different in categorizing this lesion, the frequency of appearance of mucosal breaks of healthy subjects was analyzed here.

Analysis of drug administration studies

In many studies, nonselective NSAIDs and cyclooxygenase 2-selective NSAIDs are administered to healthy subjects to compare the occurrence of small intestinal mucosal breaks. There are other studies in which mucosal protective drugs are administered at the same time as NSAIDs and aspirin to compare the effects of this combination. Capsule endoscopy has been performed in more than 1000 healthy subjects in various studies. In these studies, healthy subjects underwent capsule endoscopy before drug administration, and small intestinal evaluation of the healthy subjects was performed first. Table 1 summarizes the studies describing small intestinal mucosal breaks in healthy subjects before drug administration. Studies that do not describe these premedication small intestinal mucosal breaks and reports with a small number of subjects were not included in the Table 1.

According to the summarized results, approximately 10% of healthy subjects have small intestinal mucosal breaks. In addition, the number of mucosal breaks in subjects with mucosal breaks was approximately 1-3 in most examinations.

Analysis of small intestinal mucosal breaks in healthy subjects from our study

Our group has conducted several capsule endoscopy studies on healthy subjects. Two of these consisted of detailed tests and interviews, including tests for *Helicobacter pylori* (*H. pylori*) infection which bacterium is known to cause gastric ulcer, duodenal ulcer, atrophic gastritis, gastric cancer, *etc.*, at the start of the study. Since the number of subjects in each study was insufficient for analysis, we combined these two studies to analyze the background of healthy subjects. In two studies we tested for *H. pylori* infection with a breath test[4] and with serum antibodies[5] prior to the NSAID dosing experiment. In particular, the latter study was investigated to block randomize *H. pylori*-infected individuals evenly when randomized.

Table 2 shows the cases that were analyzed. The total number of cases included in the two studies was slightly higher than that shown in Table 1. This is because the first capsule endoscopy was completed, but the subjects who dropped out of the study were added. The subjects enrolled in the study were those who were considered to be healthy subjects after screening, the results of basic blood tests and physical tests were normal, and were not taking any medication. The number of mucosal breaks in the small intestine determined by capsule endoscopy in these healthy subjects was 0.24 ± 1.21 (mean ± SD). Based on these results, if the average ± 2 SD of healthy subjects is considered to be the normal range, up to 2 mucosal breaks of the small intestine are in the normal range.

Next, since the number of mucosal breaks recognized in the subjects was 1-2 in most cases, subjects were divided into 21 cases with mucosal breaks and 201 cases without mucosal breaks. Then, sex, age, body mass index (BMI), smoking, alcohol consumption, and *H. pylori* infection were compared and analyzed. The results of the univariate analysis showed that the incidence of mucosal breaks was higher in men, in subjects that had a higher BMI, and in *H. pylori*-infected subjects. Therefore, when multivariate analysis was performed with these factors, mucosal breaks were strongly correlated with BMI and were significantly more common in *H. pylori*-infected subjects (Table 3).

MUCOSAL BREAKS ARE SIGNIFICANTLY ASSOCIATED WITH BMI AND *H. PYLORI* INFECTION

The results of this analysis revealed that small intestinal mucosal breaks were

Table 1 Reports of pretest small intestinal mucosal breaks in normal volunteers

Country	Year	No.	Subjects with mucosal breaks		Mean number of mucosal breaks		Ref.
			Number	%	Subjects with MB	All subjects	
United States	2005	413	57[1]	13.8[1]	N/A	N/A	Goldstein *et al*[10], 2005
United States	2007	472	56	11.9	N/A	N/A	Goldstein *et al*[11], 2007
Japan	2009	32	3	9.4	1.7	0.16	Fujimori *et al*[12], 2009
Canada	2009	20	0	0	0	0	Smecuol *et al*[13], 2009
Japan	2010	20	2	10.0	1-2	0.1-0.2	Shiotani *et al*[14], 2010
Japan	2011	72	4	5.6	1.0	0.56	Fujimori *et al*[4], 2011
Japan	2013	32	N/A	N/A	N/A	0.1-0.3	Kuramoto *et al*[15], 2013
China	2014	30	2	6.7	3.5	0.23	Huang *et al*[16], 2014
Japan	2014	30	N/A	N/A	N/A	0.14 ± 0.54	Umegaki *et al*[17], 2014
Japan	2016	141	14	9.9	3.5	0.35	Fujimori *et al*[5], 2016
Japan	2015	37	N/A	N/A	N/A	0.1-0.3	Kojima *et al*[18], 2015
Czech	2016	42	2	4.7	N/A	N/A	Tachecí*et al*[19], 2016
Japan	2016	19	2	10.5	1.0	0.11	Arimoto *et al*[20], 2016
Japan	2016	45	N/A	N/A	N/A	0.13	Ota *et al*[21], 2016
Japan	2019	24	0	0	0	0	Ota *et al*[22], 2019

[1]Lesion type unknown. Reports with 15 or more subjects and reports on premedication lesions were collected. MB: Mucosal break; N/A: Not available.

Table 2 Basic background of the two examinations whose background was examined in detail

Ref.	Number	Sex (M/F)	Age (mean ± SD)	Mean mucosal break
Fujimori *et al*[4], 2011	77	77/0	38.2 ± 8.1	0.12 ± 0.32
Fujimori *et al*[5], 2016	145	71/74	48.6 ± 6.4	0.30 ± 1.47
Total	222	148/74	45.0 ± 8.6	0.24 ± 1.21

Premedication capsule endoscopy was completed, and subjects who dropped out in subsequent studies were included.

correlated with BMI. In many reports, it has been reported that obese people have different intestinal flora compared to those with proper weight. Additionally, specific bacteria, which are predominantly present in obese people, cause tight junctions in the intestinal tract to malfunction allowing bacterial products to pass through the intestinal wall[6]. The results of the analysis are considered to support this idea. High-fat diet also affects gut flora[7]. In addition, blood flow disorders due to arteriosclerosis and lack of exercise may be involved.

In addition, small intestinal mucosal breaks was higher in *H. pylori*-infected subjects. We do not consider *H. pylori*-infected subjects to be completely healthy individuals, so *H. pylori* infection was investigated prior to the capsule endoscopy study to evenly distribute *H. pylori*-infected subjects between the two groups in our previous study[5]. The results of this study support the idea that *H. pylori* infection also affects the small intestine. Currently, it is easy to understand that the reason why *H. pylori*-infected individuals have many small intestinal mucosal breaks is that *H. pylori* infection causes atrophic gastritis, which is caused by changes in the intestinal flora due to a decrease in gastric acid concentration. Experimental reports of exacerbation of NSAID ulcers by administration of proton pump inhibitors (PPIs) are widely known[8]. Since the bacterial cell components and bacterial products of *H. pylori* flow in the lumen of the small intestine and their involvement is doubtful, such examination may be necessary in the future.

Table 3 Analysis of mucosal breaks and background factors

Valuable	Mucosal break + (*n* = 21)	Mucosal break – (*n* = 201)	*P* value[1]	*P* value[2]
Sex	Male/female: 19/2	Male/female: 129/72	0.015	0.13
Age	44.0 ± 6.7	45.2 ± 8.8	0.54	
BMI	24.7 ± 3.6	22.5 ± 3.0	0.022	0.0074
Smoking	5 (23.8%)	56 (27.9%)	0.69	
Drinking	14 (66.7%)	140 (70.0%)	0.78	
Helicobacter pylori	8 (38.1%)	13 (6.4%)	0.054	0.035

[1]Sex, smoking, alcohol consumption, and *Helicobacter pylori* were analyzed by the chi-square test, and age and body mass index were analyzed by the Mann-Whitney *U* test.
[2]*P* value by logistic multivariate analysis. BMI: Body mass index.

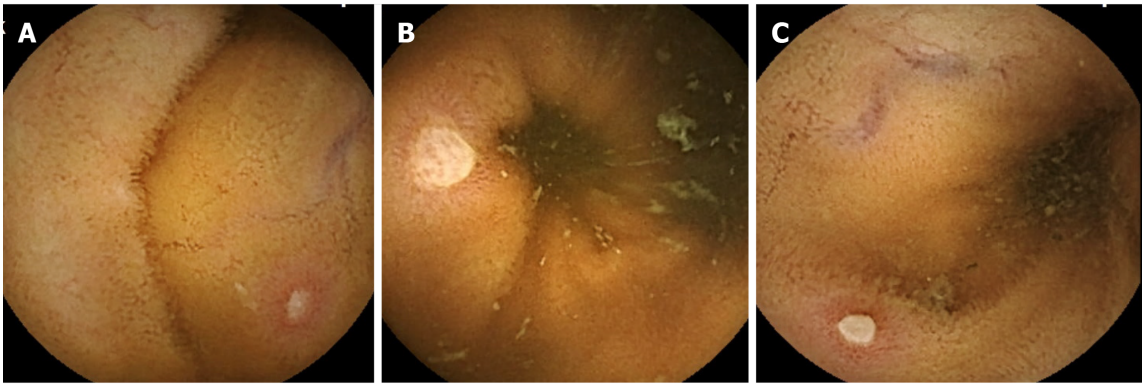


Figure 1 Endoscopic findings of small intestinal mucosal breaks. A: Erosion with white slough of approximately 3 mm; B: Ulcer with white slough of approximately 7 mm; C: The white slough is approximately 5 mm, and opinions of evaluation are divided by researchers.

In addition, small intestinal mucosal breaks tended to be higher in men. We were doubtful that smoking and alcohol consumption were related to mucosal breaks and no relationship was found between them in the analysis. CD is more common in men, and men may have some sex differences that are more likely to cause ulcerative lesions in the small intestine.

WHAT TO DO WITH SMALL INTESTINAL MUCOSAL BREAKS?

Any obvious ulcer should be considered abnormal. Table 4 summarizes the diseases and causes that cause small intestinal ulcers. Although it is not possible to mention the ulcer size here, it is natural to consider even one ulcer that is considered to be large or deep, such as white slough exceeding 1 cm, to be abnormal. However, what should we think of a small mucosal break with white slough up to approximately 5 mm?

As mentioned above, nearly 10% of healthy people have 1-2 small mucosal breaks. It would be physically and economically burdensome to follow-up with a capsule endoscopy for a person with a small number of asymptomatic mucosal breaks. Among the subjects examined in this study (Table 2), there were 3 male subjects with 5 or more mucosal breaks, but it was only confirmed at the end of the study that there were no abnormal symptoms or abnormal blood data. On the other hand, there is a case report in which a PPI was administered to a patient who had 6 mucosal breaks by capsule endoscopy before the study, and an increase in mucosal breaks was confirmed[9]. No abnormalities were found in this subject at a follow-up examination 2 years after the study.

If 3 or more small mucosal breaks are found in the small intestine, it is considered to exceed ± 2 SD on average and should be judged to be out of the normal range. There is a possibility that some of these asymptomatic lesions may develop into other diseases, such as CD, but there are few reports at this time, and there is not enough information

Table 4 Disease and/or cause of small intestinal ulcer with multiple reports

Category	Disease and/or cause	Ref.
Inflammatory disease	Crohn's disease	Gomollón *et al*[3], 2017
	Behçet's disease	Lee *et al*[23], 2012
	polyarteritis nodosa	Perlemuter *et al*[24], 1996
	Schönlein-Henoch purpura	Nishiyama *et al*[25], 2008
Vascular disease	Ischemic enteritis	Iwai *et al*[26], 2018
Systemic disease	Amyloidosis	Tada *et al*[27], 1994
Heridetary disease	Chronic enteropathy associated with *SLCO2A1* gene	Umeno *et al*[28], 2015
Bacterial infection	*Yersinia enterocolitica*	Matsumoto *et al*[29], 1990
	Campylobacter jejuni	Duffy *et al*[30], 1980
	Salmonella typhi	Goel *et al*[31], 2017
	Salmonella typhmurium	Boyd[32], 1985
	Salmonella enteritidis	Dworkin *et al*[33], 2001
	Mycobacterium tuberculosis	Lu *et al*[34], 2020
Viral infection	Epstein-Barr virus	Watanabe *et al*[35], 2020
	Cytomegalovirus	Matsumura *et al*[36], 2020
	COVID-19	Sahu *et al*[37], 2021
Drug induced	Various drugs included NSAIDs	Scarpignato and Bjarnason[38], 2019
Immunodeficiency	Acquired immunodeficiency syndrome	Zeitz *et al*[39], 1998
	Graft-versus-host disease	Peled *et al*[40], 2016

Reference searched as much as possible for new, review, and important ones. COVID-19: Coronavirus disease-2019; NSAID: Nonsteroidal anti-inflammatory drugs.

to consider follow-up guidelines. Considering the financial and physical burden of capsule endoscopy, it cannot be stated in detail how many mucosal breaks warrant followed-up, so presently the decision is left to each medical professional. Therefore, we think it is appropriate to perform a re-examination with a capsule endoscopy, *etc.*, when anemia or similar symptoms appear during follow-up at a normal health examination.

CONCLUSION

Based on this analysis, approximately 10% of healthy individuals have small intestinal mucosal breaks, and high BMI, *H. pylori*-infected individuals, and men are more likely to present with mucosal breaks. When up to 2 small mucosal breaks of 5 mm or less are found, this is considered to be within the normal range; therefore, asymptomatic persons with mucosal breaks could be followed up at a normal annual health examination. When a person has 3 or more small mucosal breaks, it is considered to be out of the normal range, but presently a follow-up schedule cannot be considered due to insufficient information. Nevertheless, when there is no significant number of mucosal breaks, follow-up should be carried out at a normal medical examination, and if anemia occurs, a close examination policy is sufficient. When a patient has an obvious ulcer, abnormality exists; therefore, close examination is desired. However, there is not enough information at this time to consider how to treat asymptomatic small intestinal ulcers.

We also hope that it should be clarified in future whether *H. pylori* and BMI really affect small intestinal ulcerative lesions in healthy subjects. 16s rRNA or metabolite sequencing of the intestinal flora between *H. pylori*-infected and uninfected as well as between high- and low-BMI assess whether small intestinal ulcerative lesions correlate with changes in the microbial flora. These researches such as comparing the intestinal

flora of objects is considered necessary. Future studies are required to address this issue.

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GUIDELINE INTERPRETATION

Recent advances in gastrointestinal cancers

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Abstract

Gastrointestinal cancers occur in a total of eight different locations, each of them with a different standard of care. This article is not an exhaustive review of what has been published in 2020. We have concentrated on the thirteen phase III randomized studies that are practice-changing. All these studies are oral presentations which have been given in one of the four major oncology congresses, namely American Society of Clinical Oncology (ASCO), ASCO gastrointestinal (GI), European Society of Medical Oncology (ESMO) and ESMO-GI. We provide a concise view of these major trials and their main outcomes, and put these results into context.

Key Words: Practice changing trials; Immunotherapy; Gastrointestinal cancers; Gastrointestinal; Gastric cancer

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Core Tip: Gastrointestinal cancers are one of the most frequent cancers and a leading cause of cancer deaths worldwide. Gastroenterologists, hepatologists and visceral surgeons interact daily with these patients. Bringing the community up to speed with new treatment paradigms in gastrointestinal oncology could improve patient care. Here we provide a clear, comprehensive and short overview of the most important practice changing trials from 2020.

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INTRODUCTION

Gastrointestinal cancers are the most common form of cancer, affecting both men and women. A multidisciplinary approach is critical to ensure the most effective treatment and best care for each individual patient. 2020 has been a significant year with regard to gastrointestinal cancers, with many practice-changing trials taking place. Here, we aim to provide a clear, comprehensive and brief overview of highlights in the care of gastrointestinal cancer in 2020.

GASTROEOSOPHAGEAL CANCER

Neoadjuvant

Human epidermal growth factor receptor 2 (HER2) inhibition by trastuzumab and chemotherapy has shown clinically and statistically significant improvements in disease-free survival (DFS) and overall survival (OS) in HER2-positive advanced gastroesophageal cancer compared to chemotherapy alone and is now the standard of care for these patients[1].

NRG-RTOG 1010, presented at American Society of Clinical Oncology (ASCO) 2020, was an open-label randomized phase III trial including 203 patients with newly diagnosed HER2-positive (immunohistochemistry 3+ or FISH positive) esophageal cancer (EC) or gastroesophageal junction (GEJ) cancer[2]. Patients were randomized to receive trimodal standard of care (chemotherapy, radiotherapy and surgery) with or without trastuzumab. Participants in the standard of care arm received chemo-radiation therapy (CXRT) for 6 wk followed by surgery. Participants on the experimental treatment arm received weekly neoadjuvant trastuzumab during CXRT followed by adjuvant trastuzumab for 13 treatments after surgery. The primary endpoint of the trial was DFS. The median DFS was 14.2 mo in the standard of care arm and 19.6 mo in the experimental arm with a hazard ratio (HR) 0.97 (0.69, 1.36) and P = 0.85. OS was similar in both arms, 38.9 mo *vs* 38.5 mo in the experimental arm. Pathological complete response was equivalent in both arms: 29% *vs* 27% in the experimental arm.

Impact: HER2 inhibition is currently not recommended in the neoadjuvant setting for localised HER-2 amplified EC or GEJ cancer.

CXRT improves local control and survival in EC; however, local recurrence will occur in 50% of patients.

The ARTDECO trial presented at ASCO-GI 2020 was a randomised phase III multicentre study which randomised 260 patients with locally advanced unresectable EC (93%) and GEJ cancer (7%)[3]. Patients were treated with a standard dose of 50.4 Gy/1.8 Gy/5.5 wk to the tumor and regional lymph nodes *vs* the same dose combined with an integrated boost of 0.4 Gy per fraction (total 61.6 Gy) to the primary tumor. Chemotherapy consisted of 6 weekly concurrent carboplatin and paclitaxel in both arms. The primary endpoint was local progression-free survival (LPFS). There was no statistical difference in 3-year LPFS with 71% in the standard arm *vs* 73% in the experimental arm, nor was there a statistically significant difference in OS, with 41% *vs* 40% for the standard arm and experimental arm, respectively. LPFS was similar per histology. Overall grade 4 and 5 toxicity was 12% and 4% in the standard arm *vs* 14% and 10% in the experimental arm.

Impact: Radiation dose escalation in definitive CXRT treatment for locally advanced EC does not improve local control or OS.

Adjuvant

The risk of recurrence after neoadjuvant CXRT followed by surgery remains high, > 50% during the first 2 years in EC and GEJ cancer[4]. Currently there is no established adjuvant treatment in this setting. Nivolumab — an anti-PD1 checkpoint inhibitor — demonstrated superior survival in previously treated unresectable advanced or recurrent esophageal squamous cell carcinoma (ESCC) *vs* chemotherapy (ATTRACTION-3 trial)[5] and in gastric cancer (GC)/EGJ cancer *vs* placebo (ATTRACTION-2 trial)[6].

The CheckMate 577 study presented at European Society of Medical Oncology (ESMO) 2020 and recently published in the New England Journal of Medicine was a randomised, double-blind phase III study, evaluating nivolumab *vs* placebo in the adjuvant setting after trimodal therapy for EC and GEJ cancer[7]. Following R0 resection, 794 patients with stage II (approximately 35%) or III (approximately 65%), EC (approximately 60%) and EGJ cancer (approximately 40%) and residual pathologic disease were randomised to receive nivolumab for one year or placebo. Most patients (approximately 70%) had adenocarcinomas. The primary endpoint was DFS. At a pre-specified interim analysis, adjuvant nivolumab showed an improved DFS of 22.4 mo compared to 11 mo in the placebo arm with HR = 0.69 (0.56-0.86) and P = 0.0003. Treatment-related adverse events (TRAEs) occurred in 13% of the nivolumab arm *vs* 6% in the placebo arm (Figure 1), patient-reported outcomes were similar in both arms.

Impact: Nivolumab is the first adjuvant therapy to provide a reduction in the risk of recurrence or death in resected EC/GEJ cancer with residual disease after trimodal treatment. These results establish nivolumab as a new standard of care in these patients.

Advanced/metastatic

Patients with inoperable or metastatic GC have a median OS of less than 1 year[8,9]. Currently, these patients are treated with doublet chemotherapy which has improved OS and quality of life (QoL) compared to single-agent chemotherapy or best supportive care alone[10,11].

The CheckMate 649 trial, presented at ESMO 2020, evaluated in an open label, randomised phase III trial the efficacy of nivolumab in the treatment of previously untreated, unresectable advanced or metastatic GC/EGJ cancer and esophageal adenocarcinoma[12]. A total of 1581 patients were treated with nivolumab plus chemotherapy (FOLFOX or XELOX) or chemotherapy alone. Programmed cell death ligand 1 (PD-L1) protein expression in gastric or GEJ adenocarcinoma is determined by using the combined positive score (CPS), which is the number of PD-L1 staining cells (tumour cells, lymphocytes, macrophages) divided by the total number of viable tumour cells, multiplied by 100. The specimen should be considered to have PD-L1 expression if CPS ≥ 1. Primary outcomes were OS and PFS in patients with CPS > 5%. OS in CPS > 5% was 14.4 mo in the nivolumab and chemotherapy arm *vs* 11.1 mo in the chemotherapy arm alone with HR: 0.71 (0.59-0.86, P < 0.0001) (Figure 2A). PFS in CPS > 5% was 7.7 mo in the experimental arm *vs* 6 mo (P < 0.0001) in the control arm (Figure 2B). A statistically significant benefit was also seen with nivolumab when assessing OS and PFS in patients with PD-L1 CPS ≥ 1% and the overall randomised population. Serious TRAEs occurred in 22% of the experimental arm *vs* 12% in the control arm.

The ATTRACTION-4 trial, presented at ESMO 2020, was a double-blind, phase III randomised trial evaluating the combination of nivolumab with chemotherapy (SOX or CAPOX) *vs* chemotherapy alone in GC/EGJ cancer[13]. Primary outcomes were PFS and OS. At an interim-analysis, median PFS was improved in the experimental arm at 10.45 mo *vs* 8.34 mo, HR: 0.68 (0.51-0.90) and P = 0.0007. However, OS was comparable in both arms 17.45 mo *vs* 17.15 mo in the experimental arm (P = 0.257).

The Keynote 590 study, presented at ESMO 2020, was a double-blind, placebo-controlled study evaluating pembrolizumab (anti-PD-1) plus chemotherapy (cisplatin and 5FU) *vs* placebo plus chemotherapy as first-line treatment for locally advanced unresectable or metastatic AEC or ESCC or advanced/metastatic EGJ Siewert I adenocarcinoma[14]. Primary outcomes were OS in ESCC with CPS > 10% and whole population and PFS in the whole population. The majority of subjects (approximately 70%) had ESCC, AEC represented approximately 15% and EGJ approximately 12% of the study population. OS was significantly increased in the experimental arm in ESCC with CPS > 10% with a median OS of 13.9 mo *vs* 8.8 mo in the control arm (P < 0.0001) as well as in the ESCC overall population with OS of 12.4 mo *vs* 9.8 mo (P = 0.0006). PFS in ESCC was significantly increased in the experimental arm with 6.3 mo *vs* 5.8 mo (P < 0.0001).

Impact: The combination of PD-1 blockage (nivolumab or pembrolizumab) with chemotherapy could be considered a new standard first-line option for advanced or metastatic ESCC CPS > 10%, GC and EGJ CPS > 5%.

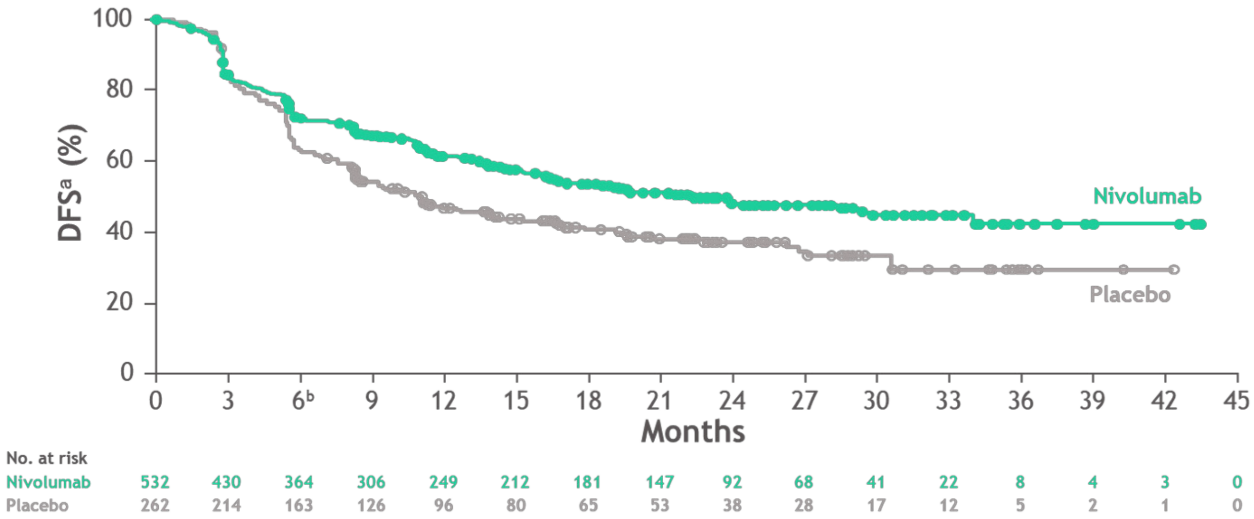


Figure 1 Disease-free survival in the study population (CheckMate 577). Citation: Kelly RJ, Ajani JA, Kuzdzal J, Zander T, Van Cutsem E, Piessen G, Mendez G, Feliciano J, Motoyama S, Lièvre A, Uronis H, Elimova E, Grootscholten C, Geboes K, Zafar S, Snow S, Ko AH, Feeney K, Schenker M, Kocon P, Zhang J, Zhu L, Lei M, Singh P, Kondo K, Cleary JM, Moehler M; CheckMate 577 Investigators. Adjuvant Nivolumab in Resected Esophageal or Gastroesophageal Junction Cancer. *N Engl J Med* 2021; 384(13): 1191-1203. Copyright ©The Authors 2021. Published by Massachusetts Medical Society[7]. DFS: Disease-free survival.

HEPATOCELLULAR CARCINOMA

Advanced/metastatic

For the last 10 years the first-line treatment of unresectable hepatocellular carcinoma (HCC) was sorafenib. In 2018, lenvatinib showed its non-inferiority compared to sorafenib, offering a second option in first-line treatment[15].

The IMbrave150 trial was an open-label, phase III trial. Patients with unresectable HCC and without previously systemic treatment were randomly assigned to receive either atezolizumab (anti-PD-L1) plus bevacizumab (anti-VEGF) or sorafenib[16]. All patients were Child-Pugh A and the aetiology of underlying liver disease was viral hepatitis in approximately 70% of the study population. The co-primary endpoints were OS and PFS. At the first interim analysis, OS at 12 mo was 67.2% in the experimental arm and 54.6% in the standard arm with HR 0.58 (0.42-0.79, $P < 0.001$). Median PFS was 6.8 mo in the experimental arm *vs* 4.3 mo ($P < 0.001$). Grade 3 or 4 adverse events occurred in 56.5% in the experimental arm and in 55.1% in the control arm. Moreover, this combination delayed the deterioration of patient-reported functioning and QoL *vs* sorafenib in this patient population to a meaningful degree.

Impact: Atezolizumab + bevacizumab is now considered first-line standard of care therapy in patients with unresectable HCC.

COLON CANCER

Adjuvant

For stage III colon cancer, the addition of oxaliplatin to a fluoropyrimidine improved DFS and OS in the adjuvant setting. Since 2004, a 6-mo regimen of FOLFOX or CAPOX is the standard adjuvant therapy in stage III disease[17-19]. However, oxaliplatin is associated with cumulative neurotoxicity that can be severe, which potentially affects patients' activities of daily living, and can persist long beyond the actual treatment. If efficacy were maintained, shorter adjuvant therapy duration would be beneficial for patients as it could spare them toxic effects and health expenditures.

An international duration evaluation of adjuvant therapy trial investigated the non-inferiority of 3 mo of adjuvant therapy (either FOLFOX or CAPOX) compared to 6 mo of treatment[20]. The primary endpoint was the rate of DFS at 3 years. Non-inferiority of 3 mo of therapy, as compared to 6 mo, was not confirmed in the overall population. However, in patients treated with CAPOX, 3 mo of therapy was as effective as 6 mo, particularly in the lower-risk subgroup.

At this year's ASCO, Sobrero *et al*[21] presented the OS (secondary outcome) and an update of the DFS (median follow-up of 6 years). Again non-inferiority of OS for 3 mo

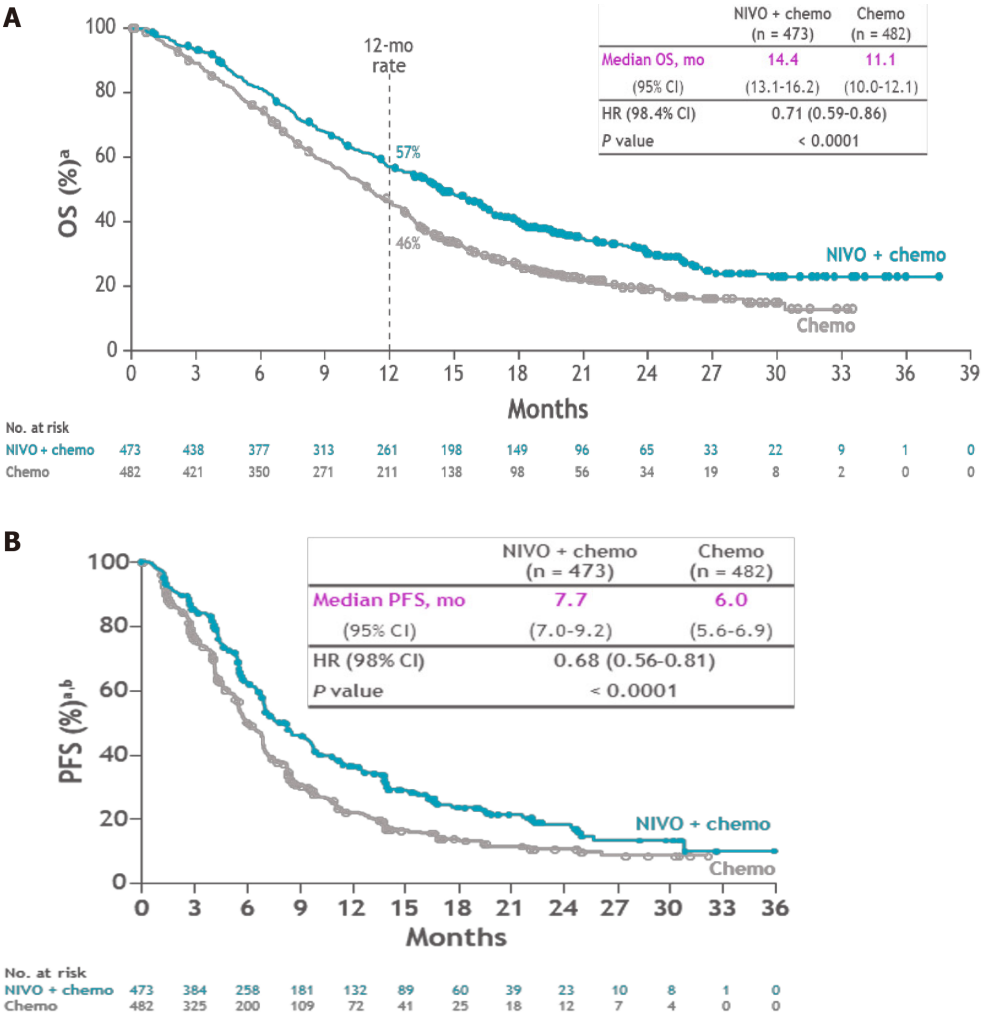


Figure 2 Overall survival and progression-free survival in patients with programmed cell death ligand 1 combined positive score of more than 5% (CheckMate 649). A: Overall survival; B: Disease-free survival. Citation: Moehler M, Shitara K, Garrido M, Salman P, Shen L, Wyrwicz L, Yamaguchi K, Skoczylas T, Bragagnoli AC, Liu T, Schenker M, Yanez P, Tehfe M, Poulart V, Cullen D, Lei M, Kondo K, Li M, Ajani JA, Janjigian YY. Nivolumab (nivo) plus chemotherapy (chemo) *vs* chemo as first-line (1L) treatment for advanced gastric cancer/gastroesophageal junction cancer (GC/GEJC)/esophageal adenocarcinoma (EAC): First results of the CheckMate 649 study. *Ann Oncol* 2020; 31(4): S1191. Copyright ©The Author(s). Published by European Society of Medical Oncology[12]. PFS: Progression-free survival; HR: Hazard ratio; CI: Confidence interval; OS: Overall survival.

(82.4%) *vs* 6 mo (82.8%) treatment was not demonstrated (P = 0.0583). OS was 82.1% for patients treated with 3 mo of CAPOX compared to 81.2% in those receiving it for 6 mo. In patients treated with FOLFOX, 3 mo survival was 82.6% compared to 83.8% for 6 mo survival (Figure 3A). Five-year DFS was 70% for 3 mo CAPOX compared to 69.3% for 6 mo and 68.4% for 3 mo FOLFOX compared to 71.7 for 6 mo (Figure 3B). Finally, for low risk (T1-3, N1) cancers, OS with 3 mo treatment was 89.6% compared to 88.9% for 6 mo. In high risk (T4 / N2) cancers, OS was 72% for 3 mo and 74.1% for 6 mo.

Impact: Stage III low risk colon cancer can be treated with either 6 mo FOLFOX or 3 mo CAPOX. High-risk stage III colon cancer can be treated with 6 mo FOLFOX or 6 mo CAPOX, although the difference in clinical benefit between 3 and 6 mo CAPOX is small.

Follow-up

In colon cancer, 30%-50% of all patients will relapse and die of the disease. Intensive follow-up of patients after curative surgery to detect recurrence and/or metachronous cancers is recommended[22]. However, these recommendations are based on expert opinions, and clinical trials have shown contrasting results[23]. A Cochrane meta-analysis showed no survival benefit of intensive follow-up[24]. Which tests should be performed and what the optimal frequency for surveillance of cancer recurrence might be are still unknown.

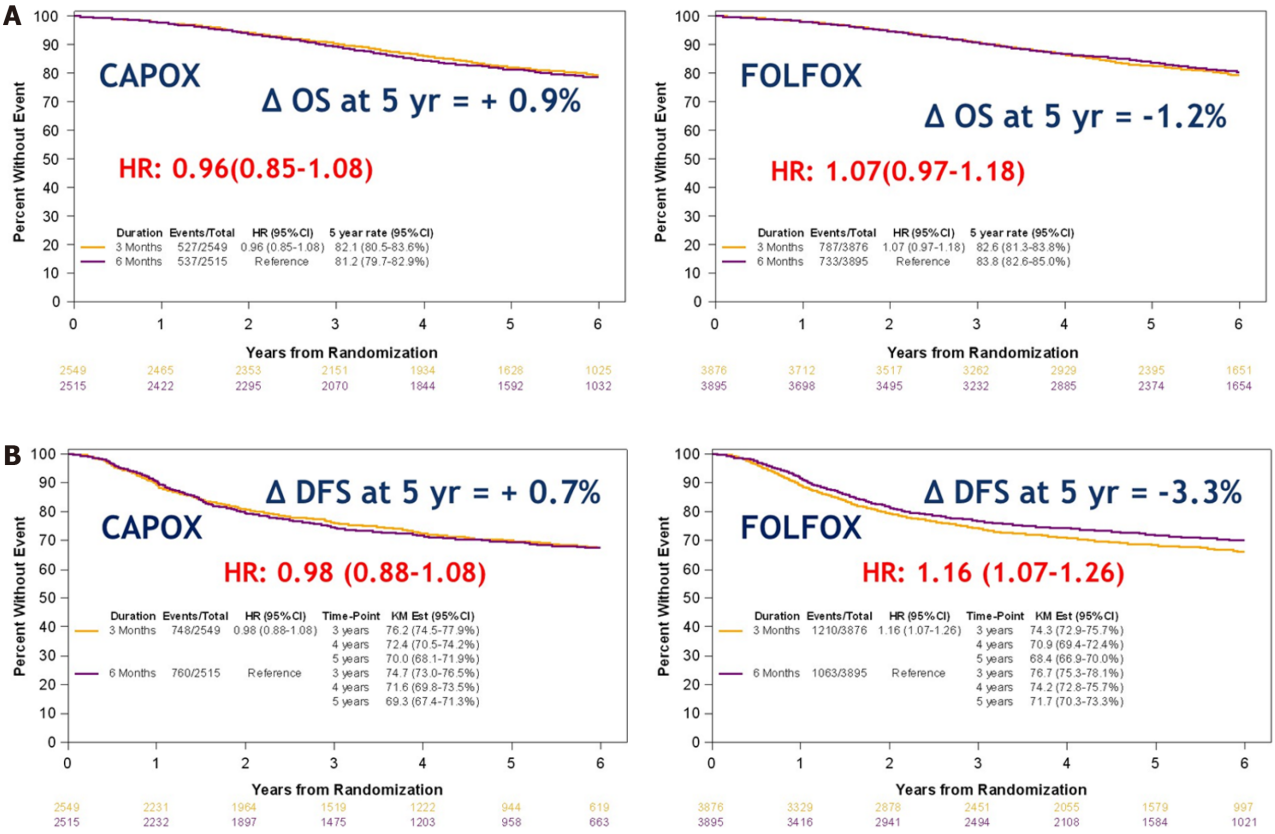


Figure 3 Overall survival and disease-free survival with 3 mo *vs* 6 mo of adjuvant therapy (international duration evaluation of adjuvant therapy). A: Overall survival; B: Disease-free survival. Citation: Sobrero AF, Andre T, Meyerhardt JA, Grothey A, Iveson T, Yoshino T, Sougklakos I, Meyers JP, Labianca R, Saunders MP, Vernerey D, Yamanaka T, Boukovinas I, Oki E, Georgoulias V, Torri V, Harkin A, Taieb J, Shields AF, Shi Q. Overall survival (OS) and long-term disease-free survival (DFS) of three *vs* six months of adjuvant (adj) oxaliplatin and fluoropyrimidine-based therapy for patients (pts) with stage III colon cancer (CC): Final results from the IDEA (International Duration Evaluation of Adj chemotherapy) collaboration. *J Clin Oncol* 2020; 38(15): 4004-4004. Copyright ©The Author(s). Published by the American Society of Clinical Oncology[21]. DFS: Disease-free survival; HR: Hazard ratio; OS: Overall survival.

The PRODIGE 13 study, presented at ESMO 2020, was a prospective multicentre phase III-controlled trial evaluating the impact of intensive follow-up on OS in 1995 resected stage II or III colorectal cancer (CRC) patients[25]. Patients were double randomised, first in the CEA assessment arm *vs* no assessment and then in the intensive radiological follow-up arm [computed tomography (CT)-scan every 6 mo] *vs* low intensity follow-up (abdominal ultrasound every 3 mo and thoracic radiography every 6 mo). The primary endpoint was 5-year OS.

The majority (77%) of patients were less than 75 years old and had CRC (16% rectal, 84% colon cancer). Half of them had stage II disease, 50% being high risk stage II. This study showed no difference in terms of OS and recurrence-free survival for any of the surveillance arms. In the sub-group of patients with recurrence, curative surgery was achieved in 40.9% of the ''minimum follow-up'' group, 66.3% in the ''CEA'' group and standard imaging, 50.7% in the ''no CEA and CT scan'' group, and 59.5% in the maximum follow-up group. Although the differences were significant (P = 0.0035), OS was identical in all arms (P = 0.887).

Impact: A low intensity surveillance (no CEA, abdominal ultrasound and chest X-ray) could soon be the new standard for CRC surveillance after curative resection for patients not amenable to a second curative resection.

Metastatic

Patients with microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) CRC represent 5% of all patients with metastatic CRC. The presence of MSI-H is associated with decreased survival rates and less response to conventional chemotherapy[26]. However, MSI-H or dMMR tumours respond better than microsatellite stable tumours to anti-PD1 or antiPD-L1, immunotherapy. In May 2017, the FDA approved the use of pembrolizumab (anti-PD-1), for the treatment of metastatic MSI-H/dMMR CRC patients who have progressed on chemotherapy.

The KEYNOTE-177 trial presented at the plenary session at ASCO 2020 and recently published in the New England Journal of Medicine was a randomised, phase III clinical trial that evaluated the efficacy of pembrolizumab in the first-line treatment of newly diagnosed MSI-H/dMMR metastatic CRC compared to chemotherapy (doublet +/- biologic)[27]. The co-primary outcomes were PFS and OS. At a median follow-up of 32.4 mo, the median PFS with pembrolizumab was 16.5 mo compared to 8.2 mo with chemotherapy HR: 0.60 (0.45-0.80, $P = 0.0002$). Adverse events grade ≥ 3 were less common (22%) in the experimental arm compared to the standard arm (66%). QOL assessed with QLQ-C30 and EQ-5D was improved with pembrolizumab compared to chemotherapy ($P = 0.002$).

Impact: Pembrolizumab (anti-PD1) is a new standard of care in the first-line treatment of MSI-H/dMMR metastatic CRC.

Approximately 20% of patients with CRC are diagnosed with stage IV disease. For those with an asymptomatic primary tumour, the question remains whether the primary tumour should be removed first.

The iPACS study presented at ASCO-GI 2020 was a randomised phase III trial comparing primary tumour resection (PTR) plus chemotherapy compared to chemotherapy alone in asymptomatic stage IV CRC patients[28]. The primary endpoint was OS. Investigators aimed to recruit 770 patients but due to slow accrual the sample size was decreased to 280. The Data and Safety Monitoring Committee (DSMC) recommended early termination of the trial due to futility at the first interim analysis in September 2019. With a median follow-up period of almost 2 years, there was no OS difference ($P = 0.69$) between PTR plus chemotherapy (OS: 25.9 mo) and the chemotherapy alone arm (26.7 mo). Median PFS was 10.4 mo for PTR plus chemotherapy *vs* 12.1 mo for chemotherapy alone. There were three treatment-related deaths following PTR due to postoperative complications.

Impact: Currently, PTR in asymptomatic stage IV CRC patients cannot be recommended.

RECTAL CANCER

Neoadjuvant

Locally advanced rectal cancer is treated with neoadjuvant CXRT followed by surgery 8 to 12 wk later. Neoadjuvant CXRT provides a significant reduction in local recurrence but not in distant metastasis rate[29]. Furthermore, adjuvant chemotherapy in rectal cancer is difficult to administer due to poor compliance[30-32]. Indeed, rectal cancer surgery is frequently associated with a high complication rate and patients are not always able to receive chemotherapy postoperatively.

To ensure efficient treatment of both the local tumour and possible micrometastases, an alternative approach would be to administer the systemic therapy preoperatively after short-course radiotherapy (5 d). During this waiting period the patient is in a good condition to receive an optimal dose of chemotherapy. Two phase III trials (RAPIDO and PRODIGE 23) hypothesized that neoadjuvant chemotherapy will result in increased survival in locally advanced rectal cancer patients.

The RAPIDO trial, presented at ASCO 2020 and recently published in The Lancet Oncology, is a phase III randomised trial comparing standard CXRT before surgery +/- adjuvant chemotherapy (standard arm) to total neoadjuvant treatment (TNT) with short-course radiotherapy (5 × 5 Gy) followed by chemotherapy (XELOX 6 cycles or FOLFOX 9 cycles) before surgery (experimental arm)[33]. The trial accrued only patients with locally advanced rectal cancers defined as cT4a/b, extramural vascular invasion, cN2, involved mesorectal fascia, or enlarged lateral lymph nodes. The primary outcome was disease-related treatment failure (DrTF) defined as distant metastasis, locoregional failure, new primary CRC or treatment-related death. The 3-year DrTF was improved in the experimental arm at 23.7% *vs* 30.4% for the control arm ($P = 0.019$). The experimental arm showed an improved pathological complete response (28% *vs* 14%; $P < 0.001$) and reduced distant metastases (20.0% *vs* 26.8%, $P = 0.005$), but no improvement in locoregional failure rates (8.7% *vs* 6.0%, $P = 0.09$), 3-year OS (89.1% *vs* 88.8%, $P = 0.59$) or QoL.

The PRODIGE 23 trial presented at ASCO was a phase III randomised trial investigating TNT with modified (m)FOLFIRINOX (leucovorin, infusional 5-FU, irinotecan, oxaliplatin) before standard CXRT, followed by surgery and adjuvant treatment in locally advanced rectal cancer patients compared to standard CXRT followed by surgery and compulsory adjuvant treatment[34]. This trial also accrued only patients

Table 1 Overview of latest phase III trials in gastrointestinal cancers

Studies	Population	Intervention	Control	Primary endpoints	Results
Gastroesophageal cancers					
Neoadjuvant					
NRG-RTOG 1010	HER2-positive EC or GEJ	Trastuzumab + CXRT + surgery	CXRT + surgery	DFS	19.6 mo *vs* 14.2 mo (HR = 0.97)
ARTDECO	Locally advanced unresectable EC and GEJ	Standard CXRT + RT boost	Standard CXRT	3-yr LPFS	73% *vs* 71% (NS)
Adjuvant					
Checkmate-577	EC and EGJ and residual pathologic disease	Nivolumab	Placebo	DFS	22.4 mo *vs* 11 mo (HR = 0.69)
Advanced/metastatic					
Checkmate-649	Untreated, unresectable advanced or metastatic GC/EGJ	Nivolumab + CT	CT	OS; PFS	14.4 mo *vs* 11.1 mo (HR = 0.71); 7.7 mo *vs* 6 mo (HR = 0.68)
ATTRACTION-4	Untreated, unresectable advanced or metastatic GC/EGJ	Nivolumab + CT	CT + placebo	OS; PFS	17.45 mo *vs* 17.15 mo (HR = 0.90); 10.45 mo *vs* 8.34 mo (HR = 0.68)
Keynote-590	Untreated, unresectable advanced or metastatic EC/EGJ	Pembrolizumab + CT	CT + placebo	OS; PFS	12.4 mo *vs* 9.8 mo (HR = 0.73); 6.3 mo *vs* 5.8 mo (HR = 0.65)
Hepatocellular carcinoma					
Advanced/metastatic					
Imbrave 150	Untreated, unresectable HCC	Atezolizumab + bevacizumab	Sorafenib	12-mo OS; PFS	67.2% *vs* 54.6% (HR = 0.58); 6.8 mo *vs* 4.3 mo (HR = 0.59)
Colon cancer					
Adjuvant					
IDEA	Resected stage III CRC	3 mo adj. CAPOX 3 mo adj. FOLFOX	6 mo adj. CAPOX ; 6 mo adj. FOLFOX	5-yr DFS	70% *vs* 69.3% (HR = 0.95); 68.4% *vs* 71.7% (HR = 1.16)
Follow-up					
PRODIGE 13	Resected stage II or III CRC	Low intensity follow-up	High intensity follow-up	5-yr OS	NS
Metastatic					
Keynote-177	Untreated, unresectable metastatic MSI-H/dMMR CRC	Pembrolizumab	CT	PFS	16.5 mo *vs* 8.2 mo (HR = 0.6)
iPACS	Asymptomatic stage IV CRC	Tumor resection + CT	CT	OS	25.9 mo *vs* 26.7 mo (HR = 1.10)
Rectal cancer					
Neoadjuvant					
RAPIDO	Locally advanced rectal cancer	RT + Neoadj. CT + surgery	CXRT + surgery +/-adj. CT	3-yr DrTF	23.7% *vs* 30.4% (HR = 0.76)
PRODIGE 23	Locally advanced rectal cancer	Neoadj. CT + CXRT + surgery + adj. CT	CXRT + surgery +/-adj. CT	3-yr DFS	75.7% *vs* 68.5% (HR = 0.69)

CRC: Colorectal cancer; CT: Computed tomography; CXRT: Chemo-radiation therapy; DFS: Disease-free survival; dMMR: Mismatch repair deficient; DrTF: Disease-related treatment failure; EC: Esophageal cancer; GC: Gastric cancer; GEJ: Gastroesophageal junction cancer; HCC: Hepatocellular carcinoma; HR: Hazard ratio; LPFS: Local progression-free survival; MSI-H: Microsatellite instability high; NS: Not significant; OS: Overall survival; PFS: Progression-free survival; RT: Radiotherapy.

with locally advanced rectal cancers defined as cT3 or cT4 M0. The primary endpoint

was DFS. Surgical morbidity did not differ between the two arms. TNT significantly improved pathological complete response rate at 27.5% compared to 11.7% in the CXRT alone arm (P = 0.001). TNT significantly improved 3-year DFS (75.7% *vs* 68.5%, HR 0.69, P = 0.034) and 3-year metastasis-free survival (78.8% *vs* 71% in CXRT alone, HR 0.64, P = 0.02). Global quality-of-life scores were similar.

Impact: TNT could be an option in locally advanced rectal cancer patients.

CONCLUSION

This year has been particularly significant in gastrointestinal oncology (Table 1). Immunotherapy has finally entered the scene with numerous indications - first-line MSI-H/dMMR metastatic CRC, first-line metastatic ESCC CPS > 10%, as adjuvant treatment in GC with CPS > 5%, as adjuvant treatment in resected EC/GEJ cancer with residual disease after trimodal treatment and first-line advanced HCC. The field has reached maturity with treatment de-escalation in the adjuvant setting for CRC and in the follow-up after curative surgery for CRC. On the other hand, in locally advanced rectal cancer intensification with TNT has found its place in the treatment landscape.

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REVIEW

Gastrointestinal and hepatic diseases during the COVID-19 pandemic: Manifestations, mechanism and management

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is considered the causative pathogen of coronavirus disease 2019 (COVID-19) and has become an international danger to human health. Although respiratory transmission and symptoms are still the essential manifestations of COVID-19, the digestive system could be an unconventional or supplementary route for COVID-19 to be transmitted and manifested, most likely due to the presence of angiotensin-converting enzyme 2 (ACE2) in the gastrointestinal tract. In addition, SARS-CoV-2 can trigger hepatic injury *via* direct binding to the ACE2 receptor in cholangiocytes, antibody-dependent enhancement of infection, systemic inflammatory response syndrome, inflammatory cytokine storms, ischemia/reperfusion injury, and adverse events of treatment drugs. Gastrointestinal symptoms, including anorexia, nausea, vomiting, and diarrhea, which are unusual in patients with COVID-19, and some digestive signs may occur without other respiratory symptoms. Furthermore, SARS-CoV-2 can be found in infected patients' stool, demonstrating the likelihood of transmission through the fecal-oral route. In addition, liver function should be monitored during COVID-19, particularly in more severe cases. This review summarizes the evidence for extra-pulmonary manifestations, mechanisms, and management of COVID-19, particularly those related to the gastrointestinal tract and liver.

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Core Tip: The coronavirus disease 2019 (COVID-19) pandemic has affected millions worldwide, with high lethality. In addition to typical respiratory symptoms of COVID-19, gastrointestinal and hepatic symptoms have been frequently observed in COVID-19 patients. The main purpose of this article is to focus on the manifestations, mechanisms, and management of the gastrointestinal tract and liver that occurred during the course of COVID-19. Therefore, physicians must not undervalue the digestive symptoms during COVID-19 and rapidly adjust the treatment options for COVID-19 patients with gastrointestinal symptoms and liver enzyme abnormalities.

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INTRODUCTION

Coronaviruses (CoV) is the largest group of spike-like viruses in the Nidovirales family. In the last two decades the CoVs have caused three worldwide outbreaks, with the most recent pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which has resulted in coronavirus disease 2019 (COVID-19). The first 21st century outbreak in Guangdong Province, China, in November 2002, caused by SARS-CoV (SARS-CoV-1), an extreme SARS, resulted in 8098 fatalities (9.6%) globally [1,2]. The second outbreak in Saudi Arabia in 2012 was caused by Middle East respiratory syndrome (MERS)-CoV, with 2521 (36%) confirmed deaths[3].

SARS-CoV-2 first originated in China in December 2019 and critically threatened worldwide health[4,5]. On February 12, 2021, 108 million cases and 2 million deaths have been recorded in over 219 countries and regions worldwide[6]. The lung is the primary organ involved in COVID-19 pneumonia, and most COVID-19 patients suffer typical respiratory symptoms (*e.g.*, dyspnea, cough with sputum production, fatigue, and in severe cases, acute respiratory distress syndrome (ARDS), respiratory failure, and even death). On the other hand, extrapulmonary clinical manifestations of COVID-19 affect multiple other organs including cardiovascular (*e.g.*, arrhythmias, myocarditis, pericarditis, acute coronary syndrome, and heart failure), renal (*e.g.*, acute kidney injury and acute tubular necrosis), hepatic [*e.g.*, elevated alanine aminotransferase (ALT), aspartate aminotransferase (AST), and bilirubin], gastrointestinal (*e.g.*, diarrhea, nausea, vomiting, and abdominal pain), ocular (*e.g.*, epiphora, conjunctivitis, and chemosis), dermatologic (*e.g.*, erythematous rash, urticaria, and chickenpox-like vesicles), and neurological systems (*e.g.*, headache, neuropathy, encephalopathy, cerebrovascular disorders, and dizziness)[7] (Figure 1). Considering all the previously mentioned data, this article examines the effects of gastrointestinal (GI) and hepatic symptoms, their associated mechanisms, and management caused by SARS-CoV-2 infection and provides a guide for clinical prevention and treatment (Figure 2).

DISEASE COURSE OF COVID-19

For SARS-CoV-2, the incubation period is an average of 4-5 d, with most patients having symptoms before 14 d, although there have been instances where the incubation period was longer[8]. The infection and hospitalization onset was documented from 9.1 to 12.5 d and emphasizes the difficulty in the early stage of the diagnosis and isolation of populations[9]. The average period for recovering patients from initial symptoms is 22 d, and for those who succumbed the time to death is approximately

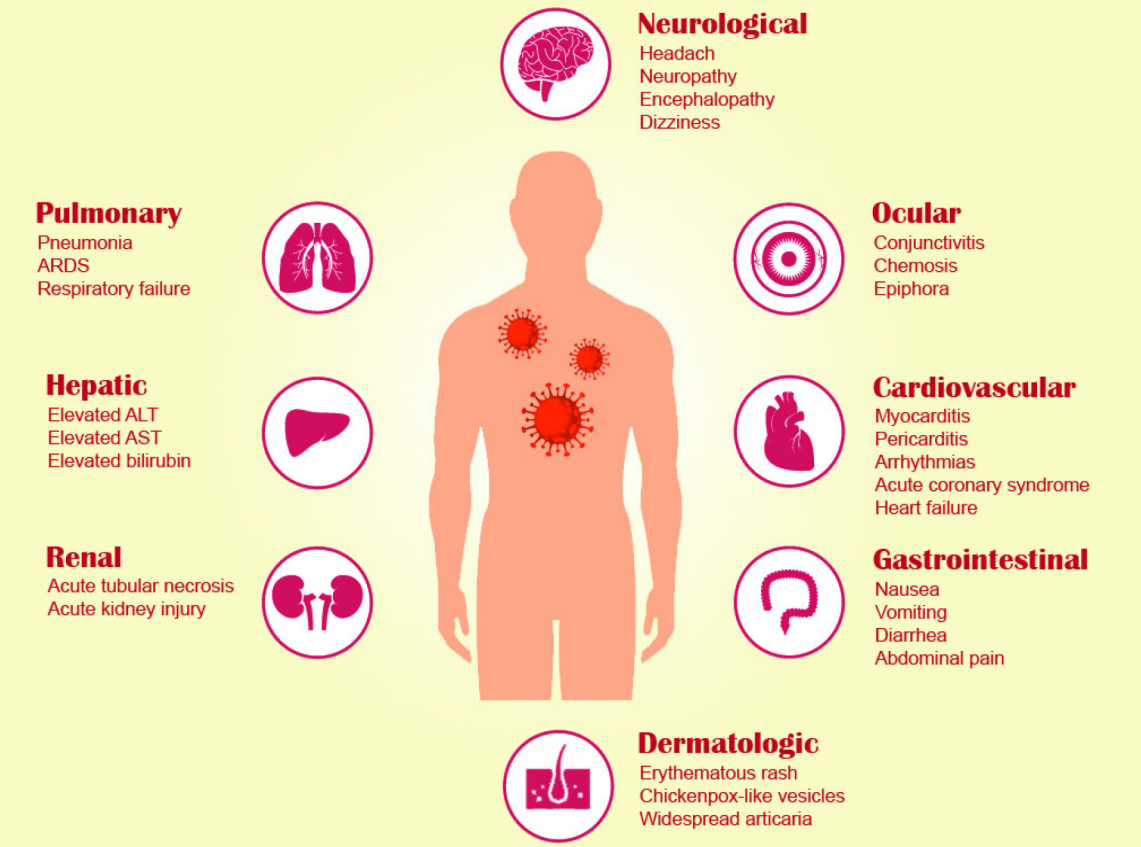


Figure 1 Pulmonary and extrapulmonary manifestations of coronavirus disease 2019. ARDS: Acute respiratory distress syndrome; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase. Headache and urticaria spelled wrongly.

18.5 d[9].

Overall, the case fatality ratio of COVID-19 is reported to be around 1-2% in patients aged 80 years, to over 15%[10]. Currently, WHO data show that most COVID-19 cases have mild to moderate signs of illness (80%), and 13.8% of cases have serious signs within 24-48 h with the following symptoms: shortness of breath; hypoxia < 300% and/or pulmonary infiltration > 50%; tachypnea > 30 breaths/min[11]. 6.1% of patients with critical infections also have septic shock, respiratory and multiple-organ failure[11,12]. Approximately 25% of hospitalized patients require intensive care unit (ICU) care, and 4.3% die[12].

GENDER AND RACE DURING COVID-19

It has been demonstrated that 51% of reported cases of COVID-19 are male patients. This may be due to the higher levels of estrogen in female COVID-19 patients which can reduce COVID-19 severity and mortality *via* the elevation in innate and humoral immunity[13-19]. Moreover, *in vivo* studies have demonstrated that there are higher levels of angiotensin-converting enzyme 2 (ACE2) expression in male kidneys than in female kidneys, which may explain the differences in the susceptibility and development of COVID-19 between male and female patients.

Whether ACE2 expression varies in the lungs of male and female COVID-19 patients, is still unclear[20,21]. Furthermore, preclinical trials have suggested that ACE2 expression may increase vulnerability to COVID-19 in pregnant patients[22,23].

In the same way, COVID-19 varies between different ethnic groups in terms of severity and mortality. During the COVID-19 pandemic, American, Hispanic, and African communities have displayed higher rates of infection and hospitalization in comparison to Caucasian communities[24]. These discrepancies may be due to the higher occurrence of heart diseases, hypertension, obesity, diabetes, and asthma in minority groups[24].

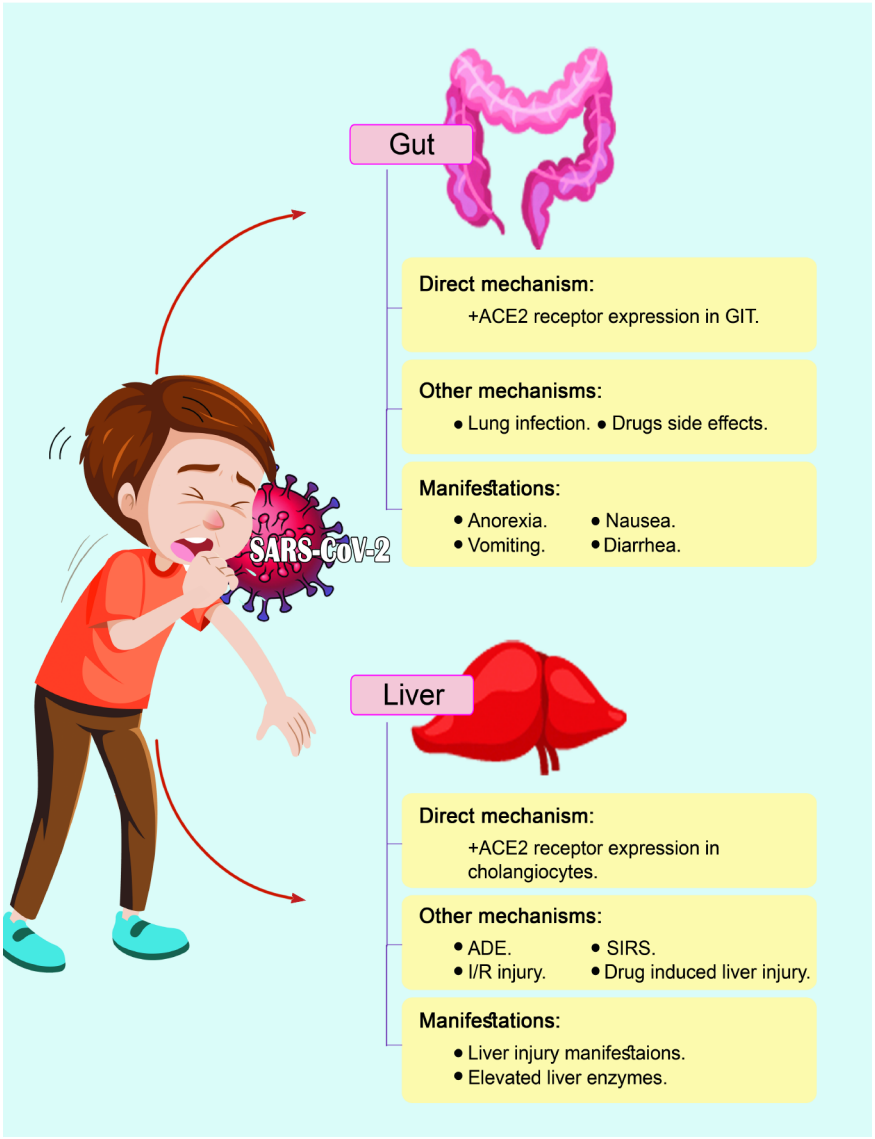


Figure 2 Mechanisms and manifestations of coronavirus disease 2019 in the gut and liver. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; ACE2: Angiotensin-converting enzyme 2; GIT: gastrointestinal tract; ADE: Antibody-dependent enhancement of infection; SIRS: Systemic inflammatory response syndrome; I/R: Ischemia and hypoxia reperfusion injury.

GASTROINTESTINAL SYMPTOMS AND COVID-19

GI manifestations in COVID-19 patients

Gastrointestinal symptoms including, nausea, anorexia, vomiting, diarrhea, and abdominal pain are common in COVID-19 patients (Table 1)[12,25-35]. In the SARS infection of 2002-2003, diarrhea was the main feature and appeared in 16%-73% of SARS patients mainly in the first week of infection[36]. Similarly, diarrhea is considered a common digestive sign in COVID-19 patients, with an incidence of 1.3%-29.3%. However, the criteria for the diagnosis of diarrhea may be different in various hospitals, and its prevalence varied in different studies[37].

Among 204 COVID-19 patients in Hubei China, only 99 (48.5%) had gastrointestinal signs as their chief complaint. These COVID-19 patients had a diversity of digestive symptoms, including abdominal pain (0.4%), anorexia (83.8%), diarrhea (29.3%), and vomiting (0.8%)[38]. A retrospective study conducted by Guan *et al*[39] reported that among 1099 COVID-19 patients from 552 centers across China, 5% of cases had nausea and vomiting, and 3.8% of cases had diarrhea. A further study indicated that 32.5% of COVID-19 patients had at least one gastrointestinal tract (GIT) symptom. These symptoms included diarrhea (37.8%), anorexia (56.7%), abdominal pain (10.4%), nausea (16.5%), and vomiting (7.9%)[40]. Additionally, Luo *et al*[41] documented that 16% of 1141 cases, had at least one GIT symptom including, anorexia (98%), nausea, vomiting (66%), diarrhea (37%), and abdominal pain (25%). Furthermore, a research

Table 1 Incidence of common gastrointestinal symptoms in patients with severe acute respiratory syndrome coronavirus 2 infection

Ref.	Patient number	Anorexia, nausea or vomiting, *n* (%)	Diarrhea, *n* (%)	Abdominal pain, *n* (%)
Kujawski *et al*[207], 2020	12	Nausea: 3 (25)	4 (33.3)	2 (16.7)
Hajifathalian *et al*[44], 2020	1059	Anorexia: 240 (22.7)	234 (22.1)	72 (6.8)
		Nausea: 168 (15.3)		
		Vomiting: 91 (8.6)		
Young *et al*[208], 2020	18	NA	3 (17)	NA
Tabata *et al*[209], 2020	104	NA	8 (9.6)	NA
Wölfel *et al*[210], 2020	9	NA	2 (22)	NA
Chen *et al*[26], 2020	99	Nausea and vomiting: 1 (1)	2 (2)	NA
Xu *et al*[27], 2020	62	NA	3 (8)	NA
Gritti *et al*[211], 2020	21	NA	5 (23.8)	NA
COVID-19 National Incident Room Surveillance Team[212]	295	Nausea: 34 (11.5)	48 (16.3)	6 (1)
COVID-19 National Emergency response Center[213]	28	NA	2 (7)	1 (4)
Sierpiński *et al*[214], 2020	1942	NA	470 (24.2)	NA
Wu *et al*[28], 2020	80	Nausea and vomiting: 1 (1.25)	1 (1.3)	NA
Wang *et al*[12], 2020	138	Anorexia: 55 (39.9)	14 (10.1)	3 (2.2)
		Nausea: 14 (10.1)		
Shi *et al*[29], 2020	81	Anorexia: 1 (1)	3 (4)	NA
		Vomiting: 4 (5)		
Yang *et al*[30], 2020	50	Vomiting: 2 (4)	NA	NA
Mo *et al*[31], 2020	155	Anorexia: 26 (31.7)	7 (4.5)	NA
		Nausea: 3 (3.7)		
		Vomiting: 3 (3.7)		
Qi *et al*[215], 2020	267	Anorexia: 46 (14.2)	10 (3.7)	NA
		Nausea: 6 (2.2)		
Wen *et al*[216], 2020	417	NA	29 (7)	NA
Dan *et al*[217], 2020	305	Anorexia: 101(50.2)	146 (49.5)	12 (6)
		Nausea: 59 (29.4)		
		Vomiting: 3 (2)		
Ma *et al*[218], 2020	81	NA	6(7.41)	NA
Luo *et al*[41], 2020	1141	Anorexia: NA	68 (6)	45 (3.9)
		Nausea: 134 (11.7)		
		Vomiting: 119 (10.4)		
Liu *et al*[219], 2020	238	Anorexia: 14 (9.2)	14 (9.2)	1 (0.7)
		Nausea: 2 (1.3)		
		Vomiting: 3 (2)		
Ai *et al*[220], 2020	102	Anorexia: NA	15 (14.3)	3 (2.9)
		Nausea: 9 (8.8)		
		Vomiting: 2 (2)		
Zhao *et al*[221], 2020	75	NA	7 (9.3)	1 (1.3)

Li *et al*[222], 2020	83	NA	7 (8.4)	7 (8.4)
Lin *et al*[223], 2020	95	Anorexia: 17 (17.8) Nausea: 17 (17.9) Vomiting: 4 (4.2)	23 (24.2)	2 (2.1)
Cholankeril *et al*[224], 2020	207	Anorexia: NA Nausea: 22 (10.8) Vomiting: NA	22 (10.8)	14 (7.1)
Ferm *et al*[43], 2020	892	Anorexia: 105 (11.8) Nausea: 148 (16.6) Vomiting: 91 (10.2)	177 (19.8)	70 (7.8)
Redd *et al*[225], 2020	318	Anorexia: 110 (34.8) Nausea: 84 (26.4) Vomiting: 49 (15.4)	107 (33.7)	46 (14.5)
Kluytmans *et al*[226], 2020	86	NA	16 (18.6)	5 (5.8)

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; NA: Not applicable; COVID-19: Coronavirus disease 2019.

study conducted by Jin *et al*[42], which was the first COVID-19 study outside Wuhan, reported that the incidence of GIT symptoms was 11.4%; diarrhea was the most common of these symptoms (8.14%)[42].

In New York, GIT symptoms have been identified in a single-center-case series, which included 892 cases. The most common symptom was diarrhea (19.8%), accompanied by nausea (16.6%), abdominal pain (7.8%), vomiting (10.2%), and anorexia (11.8%)[43]. An additional retrospective study conducted in New York showed that of 1059 COVID-19 patients, 22% had diarrhea, 6% had nausea, 19% had vomiting, and 7% had abdominal pain[44]. In addition to these studies, the pooled prevalence of GI symptoms was found to be 17.6 % in a meta-analysis of 60 studies involving 4243 cases in 6 countries The most frequent symptom was anorexia (26.8%), accompanied by diarrhea (12.5%), nausea/vomiting (10.2%) and stomach pain/discomfort (9.2%)[45].

Furthermore, Goyal *et al*[46] indicated that hyperlipidemia was found in 11.7% of patients affected by SARS-CoV-2 where 92 patients out of 756 with COVID-19, had elevated serum lipase levels, resulting in acute pancreatitis. Therefore, COVID-19 patients with hyperlipidemia have an approximately 3-fold higher risk of poor clinical outcomes, including the need for ICU admission, mechanical ventilation, and death [46].

The study by Gadiparthi *et al*[47] showed that the higher Glasgow Blatchford bleeding score was 7 and 11 in 2 of 3 patients on admission and represents a high risk of gastrointestinal bleeding (GIB) with a need for intervention of more than 50%. However, both young patients responded by carefully controlling hemodynamic parameters, levels of hematocrit or hemoglobin, transfusion of packed red blood cells as needed and medical therapy. Although GIB was resolved, two patients died due to respiratory failure[47].

Besides GIT symptoms, autopsies of COVID-19 patients showed that there are gross and microscopic changes in the GIT. Segmental dilation and stenosis of the small intestine, accompanied by mucosal shedding and necrosis was described at autopsy, whereas colitis, inflammatory infiltrates, and interstitial edema, were found on histopathology imaging[48,49]. Lui *et al*[50] have shown pneumatosis, intestinalis, pneumoperitoneum, ascites, and thickening bowel wall in addition to ileus on abdominal imaging. Therefore, abnormalities of the GIT can be obvious on imaging.

Based on these previous data, physicians must realize that the key characteristics of COVID-19 infection before respiratory symptoms may be digestive symptoms and, in rare instances may be the only COVID-19 symptoms to occur.

Mechanisms of GI injury during COVID-19

Direct infection of gastrointestinal cells: The entry of SARS-CoV-2 virus into host cells is the main part of cross-species transmission. All coronaviruses bind to receptors

and mediate their entry *via* glycoprotein and spike proteins[51]. It has been recognized that ACE2 mainly contains receptors for SARS-CoV[52], and dipeptidyl peptidase 4, for MERS-CoV[53]. A plethora of studies has shown that the spike (S) protein of SARS-CoV-2 has a high host ACE2 affinity[54], and enters host cells *via* the ACE2 receptor [55] as shown in Figure 3.

In small intestine cells, ACE2 is usually located and strongly expressed in type II epithelial cells[56,57]. It is considered an important enzyme in the renin-angiotensin system (RAS)[58,59] and plays an essential role in controlling inflammation and diarrhea[60]. Moreover, its deficiency leads to the accumulation of angiotensin II (ANG II)[61]. Subsequent studies[12,62-64] have proven that SARS-CoV-2, in COVID-19 patients, may invade the GIT *via* ACE2 receptors, causing digestive symptoms. It is now well acknowledged that the significant upsurge of ANG II level in COVID-19 patients is the cause of progression and severity of the disease.

Although in the mouse colon, the knockdown of ACE2 by the virus leads to an upsurge of ANG II levels; ACE2 does not primarily act *via* the intestinal RAS system but controls the homeostasis of intestinal amino acids, gut microbes and the expression of antimicrobial peptides[65]. Mice with blocked ACE2, for example, have significantly inhibited serum tryptophan that is important to the body's niacin synthesis[66], therefore, inadequate intake of tryptophan or niacin results in pellagra[65]. It is well-recognized that tryptophan is absorbed on the lumen surface of intestinal epithelial cells *via* the B0AT1/ACE2 transport route and induces the mammalian target of rapamycin, which controls the consequently appearance of antimicrobial peptides, thereby having a direct influence on components of intestinal flora[65]. Ultimately, more than 90% of pellagra patients will develop colitis[67]. Hence, it is considered that SARS-CoV-2 attachment to the ACE2 in the GIT lowers the critical receptor level, disrupting the absorption of tryptophan and ultimately destroying the steady-state of the gut flora, resulting in diarrhea.

Furthermore, He *et al*[68] demonstrated that the pathological results of autopsies on COVID-19 patients indicated that proinflammatory cytokines (PICs) such as tumor growth factor-β1, monocyte chemokine-1 (MCP-1), tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 were highly expressed in ACE2-expressing cells. There is an absence of PICs in cells that do not express ACE2. Numerous PICs produce the cytokine storm, which finally leads to multiple organ failure. Besides, plasma IL-2, recombinant human interferon (IFN)-induced protein-10, MCP-1, macrophage inflammatory protein-1A, IL-7, granulocyte colony-stimulating factor, IL-10, and TNF-α levels in COVID-19 patients were higher than those in healthy people[25]. Dendritic cells and macrophages show low levels of IFN and high levels of PICs and chemokines in the early stage of SARS-CoV infection[69-71]. Later, there is a rise in cytokine and chemokine levels, which causes severe tissue damage due to the large number of inflammatory cells, such as neutrophils and monocytes. In the same manner, SARS-CoV-2 can induce cells expressing ACE2 to release inflammatory cytokines, leading to the cytokine storm and multiple organ failure[72]. Furthermore, cytokine storms can have an effect on immune cells; lymphocytosis is known to be a common sign in severe cases of COVID-19, with a significant decline in the number of B, CD8+ and CD4+T cells as well as natural killer cells, which leads to lymph node necrosis, spleen atrophy, hepatomegaly, renal hemorrhage, necrosis, and degeneration of neurons[73].

Thromboembolic complications are being increasingly documented in COVID-19 [74]. Acute mesenteric ischemia (AMI) has been reported in severe COVID-19 patients in addition to deep venous thrombosis and pulmonary embolism[75]. It has been demonstrated that ANG II stimulates the expression of tissue factor VIII (FVIII), von Willebrand factor, and plasminogen activator inhibitor-1 by endothelial cells, resulting in a state of hypercoagulation[76,77]. Histology of the small intestine secondary to mesenteric thrombosis revealed a prominent endothelium of the submucosa with evidence of direct viral invasion of endothelial cells along with diffuse endothelial swelling with mononuclear cell infiltrate. It is understood that there is stimulation of alternate and lectin complement trajectories [C5b-9 (membrane attack complex), C4d, and mannose-binding protein-associated serine protease 2] that destroy endothelial cells[78]. SARS-CoV-2 infection also resulted in an elevation of ANG II levels and other prothrombotic proteins, which led to AMI[78-81].

Gastrointestinal damage caused by lung infection: Any change in intestinal flora constituents affects the respiratory tract, *via* the common mucous immune system. Reciprocally, any damage to the respiratory tract mainly affects the digestive tract *via* immune regulation. This effect is known as the "gut-lung axis"[82,83]. Furthermore, SARS-CoV-2 cannot be detected in the stools of COVID-19 patients with digestive symptoms and thus GIT symptoms may not be affected by the direct damage caused

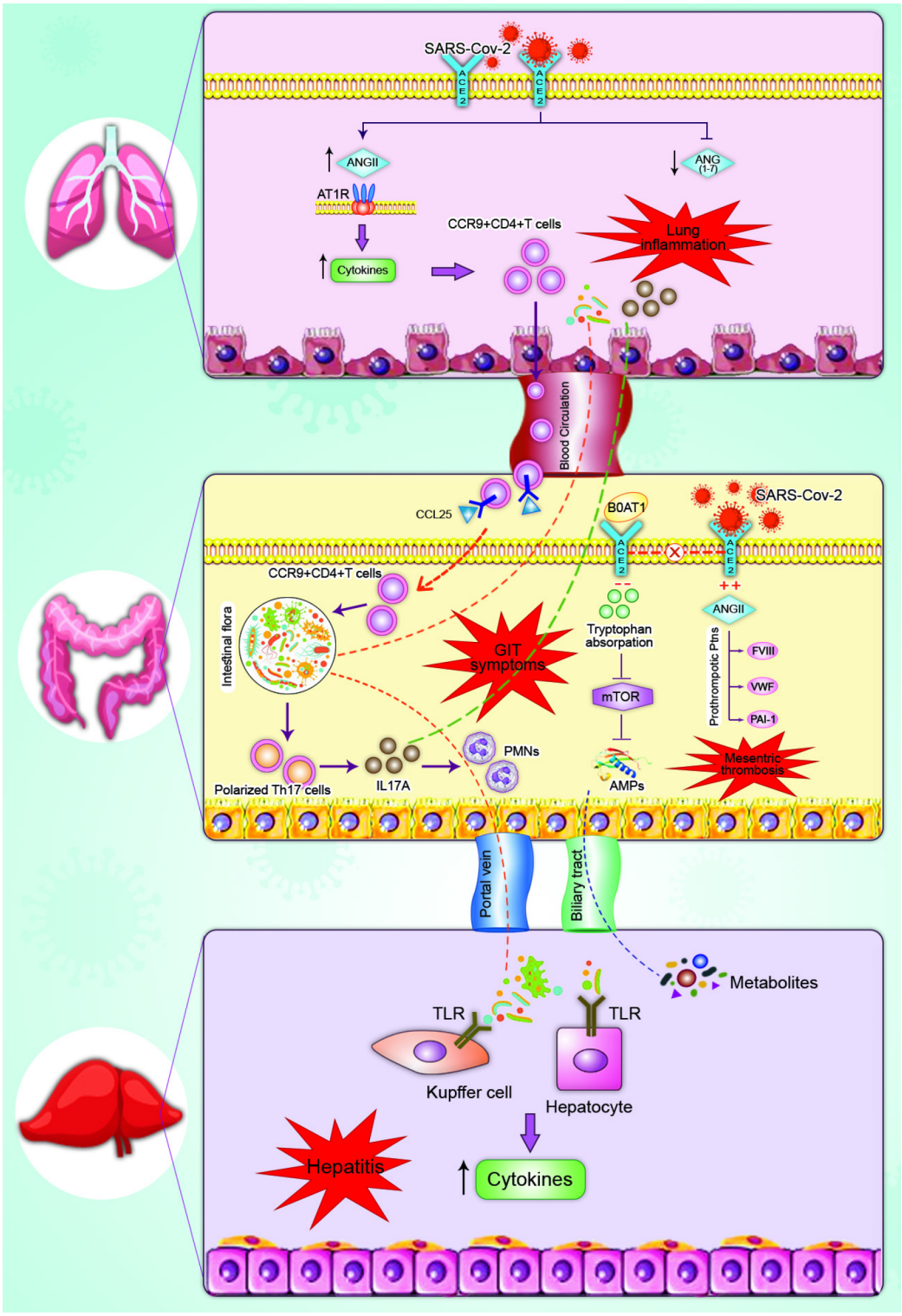


Figure 3 Mechanism of gastrointestinal symptoms in patients with coronavirus disease 2019. (1) Gut-lung axis: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) binds with angiotensin-converting enzyme 2 (ACE2) to enter the lung, which leads to the accumulation of angiotensin II (ANG II) and the reduction of Angiotensin (1-7). ANG II combined with angiotensin 1 receptor stimulates cytokine release and causes an upsurge of C-C chemokine receptor type 9 (CCR9) CD4T cells. Chemokine (C-C motif) ligand 25 subsequently enhances the recruitment of CCR9 CD4T cells into the small intestine. The changing flora then stimulates the polarization of T helper 17 cells, and eventually, interleukin 17A induces the recruitment of neutrophils. Cytokines and intestinal bacteria also enter the lung through the bloodstream, further affecting lung inflammation; and (2) Gut-liver axis: SARS-CoV-2 binds with ACE2 to enter the intestine, prevents absorption of the B0AT1/ACE2 transport pathway, and then decreases the stimulation of mammalian target of rapamycin to diminish the expression of antimicrobial peptides which result in gastrointestinal tract symptoms or enhanced ANG II that leads to the upregulation of tissue factor VIII, Von Willebrand factor, and plasminogen activator inhibitor-1 expression by endothelial cells resulting in mesenteric thrombosis. The intestinal flora, through the portal vein, is transferred to the liver, where it binds to toll-like receptors resulting in hepatitis. Additionally, the liver, can transport metabolites to the intestine *via* the biliary tract,. COVID-19: Coronavirus disease 2019; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; ACE2: Angiotensin-converting enzyme 2: ANG II: Angiotensin II; Ang1–7: Angiotensin (1-7); AT1R: Angiotensin 1 receptor; CCR9: C-C chemokine receptor type 9; CCL25: Chemokine (C-C motif) ligand 25; Th17: T helper 17; IL-17: Interleukin 17; PMNS:

Polymorphonuclear neutrophils; B0AT1: Sodium-dependent neutral amino acid transporter; mTOR: Mammalian target of rapamycin; AMPs: Antimicrobial peptides; FVIII: Tissue factor VIII; VWF: Von Willebrand factor; PAI-1: Plasminogen activator inhibitor-1; TLR: Toll-like receptor.

by SARS-CoV-2. The entry of CD4+ T cells is essential to immunity and chronic enteritis in the intestinal mucosa. It is known that C-C chemical receptor type 9 (CCR9) is an important chemical receptor for the introduction of CD4+T cells into small gut cells [84]. Wang *et al*[84] showed after viral infection, that CCR9+ CD4+ T lung derived cells were amplified. The small intestinal epithelium can include chemokine (C-C motif) ligand 25[85], which promotes the recruitment of CCR9+ CD4+ T cells into the small intestine[86], which leads to impairment of the gut's immune system and to destruction of intestinal flora homeostasis. This, in turn, stimulates the polarization of T helper 17 (Th17) cells in the intestine, and recruitment of neutrophils due to the production of large amounts of IL-17A[87], causing diarrhea, intestinal immune damage, and other gastrointestinal symptoms. Inflammation in the intestine may lead to the entry of intestinal flora and cytokines into the lung through the bloodstream, which affects the lung immune system[88]. Additionally, bacterial imbalance and intestinal mucosal damage can affect the gut-liver axis. In the intestine, host metabolites are transported through the portal vein to the liver and affect the function of the liver. The liver releases bioactive contents and bile acids and transfers them into the intestines through the systemic circulation[89]. This may lead to liver dysfunction in COVID-19 patients, due to these abnormalities[90] (Figure 3).

Gastrointestinal symptoms caused by drug side effects: Diarrhea due to antibiotics is the most common unwanted side effect of antimicrobial agents, including cephalosporins, macrolides and β-lactams. A retrospective study, in China, demonstrated that 260 COVID-19 patients treated with macrolides, fluoroquinolones, and cephalosporins resulted in 24.2% of patients developing diarrhea[91]. Another study of 138 patients with SARS-CoV-2 found that 38% had diarrhea with a medium period of 3-7 d during treatment[92]. The abovementioned data indicate that the early use of large quantities of antimicrobial agents could be linked to symptoms of diarrhea during COVID-19. Similarly, the prevalence of diarrhea in patients treated with antiviral agents such as oseltamivir is about 55.2%[93]. Other antiviral agents which can cause diarrhea as an adverse effect during the management of COVID-19 include lopinavir, chloroquine phosphate, remdesivir, and Chinese patent medicines (*e.g.*, lianhuaqingwen capsules) [94]. Additionally, treatment with broad-spectrum antibiotics is a major risk factor for *Clostridium difficile* infection, which is considered the primary reason for nosocomial diarrhea[95]. It is unclear whether the above-mentioned factors including, the use of antibiotics or antiviral agents and increased gut inflammation along with a decrease in mammalian target of rapamycin (mTOR) activity, and antimicrobial peptides, are partly responsible alone or in combination for causing diarrhea in patients infected with COVID-19 *via* alterations in the gut flora[96].

Proton pump inhibitors (PPIs) are mainly used as a therapy for patients with peptic ulcers and gastroesophageal reflux disease. By blocking the proton pump, PPIs decrease gastric acid released into the stomach. Although the decrease in stomach acid can be beneficial in patients with stomach diseases, it can make the gut more susceptible to COVID-19 infection[97-99]. SARS-CoV S protein was suggested to fuse with patients' cells in neutral pH conditions. Additionally, Darnell *et al*[100] also established that highly acidic pH (1-3) and alkaline pH (12-14) may lead to inactivation of SARS-CoV, while, in the case of neutral pH, the virus remains stable[101]. Zhou *et al* [102] reported that, under the conditions of pH 1.0 and 2.0, SARS-CoV-2 was inactivated and unable to infect cells which was related to the pH in an empty stomach by creating viruses pseudotyped with SARS-CoV-2 S protein. In addition, the study by Ramachandran *et al*[103] found that prehospitalization PPI-exposed patients had worse clinical outcomes, involving mortality of COVID-19 patients, irrespective of the existence of cardiovascular diseases. A possible explanation for this is that secretions in the stomach are in the pH range 1.0 to 3.5, while the pH in the small and large bowels ranges from 7.5 to 8.0. SARS-CoV-2 can cause gastric acid to inactivate most virus particles. If a person has a long period of acid suppression with PPIs, the pH in the stomach decreases. SARS-CoV-2 can also have a higher rate of entry in the gut, which leads to viral infection.

COVID-19 and pre-existing digestive diseases

In general, the existence of comorbidities is associated with dramatically low outcomes

in COVID-19 patients. This may cause consequences for the management of previous gastrointestinal patients[104]. Patients that have inflammatory bowel disease (IBD) and have been treated with immunosuppressive drugs are more at risk for regular and serious infections and may be more at risk for SARS-CoV-2 infection[105]. It was reported in the IBD registry in Wuhan that none of 318 patients (204 patients with ulcerative colitis and 114 patients with Crohn's disease) had been infected with SARS-CoV-2 following precautions for COVID-19 control and prevention[106]. Thus, the use of biologics and immunosuppressants, diet therapy, deliberate postponement of elective and endoscopic surgery and provisions for personal safety are recommended [107].

Considerations and management of IBD medications during COVID-19 infection: It is currently not recommended that IBD patients stop immunosuppressant drugs[11]. This is because there is a risk of disease reactivation in patients who discontinue their treatment, leading to severe inflammation, surgery, increased risk of hospitalization, and infection[108,109]. Non-hormonal anti-inflammatory drug use should be prohibited as the adverse effects of viral respiratory infections have been associated with triggering the reactivation of IBD[110].

No evidence-based recommendations for immunosuppressed patients have been made by clinical parties. Table 2 shows the specific considerations for the treatment of IBD patients mainly based on specialist beliefs. These suggestions can be edited and updated as new evidence emerges[108,110]. Overall, the same therapies can be used by patients with moderate to serious Crohn's disease or ulcerative colitis, regardless of COVID-19 infection[110]. IBD therapies can be restarted after 14 d for asymptomatic IBD patients with SARS-CoV-2 infection whose therapy has been stopped[110].

It is noteworthy that IBD patients should discontinue immunosuppressant drugs until COVID-19 is resolved, and medications can be resumed after complete resolution of COVID-19 symptoms or after two negative polymerase chain reaction (PCR) tests [111].

IBD surgery in the context of the COVID-19 pandemic: The new strain of coronavirus has increased the risk of severe pulmonary infection and extended hospitalization for IBD patients waiting for surgery, which is critical for IBD immunosuppressed patients. A recent report in Wuhan, China, demonstrated that 34 patients who underwent elective surgical procedures during the COVID-19 incubation period, developed COVID-19 pneumonia after surgery; 44.1% of patients were admitted to the ICU, and 20.5% of patients died[112]. Therefore, the majority of physicians have recommended postponing both elective and endoscopic surgery to protect patients and healthcare personnel and to minimize the usage of healthcare services and use personal protection equipment[113,114]. Eventually, the ultimate goal is to ensure the safety of patients and health care workers.

COVID-19 and the fecal–oral transmission route

A number of articles have disclosed that SARS-CoV-2 RNA can be found in patients' stool, suggesting that SARS-CoV-2 has the ability to be transmitted *via* the fecal-oral route[115-117]. It has been demonstrated that some COVID-19 patients had positive fecal but negative PCR tests[118]. Wang *et al*[117] revealed that of 153 (29%) patients with COVID-19, 44 showed positive stool virus. Moreover, Xiao *et al*[49] indicated that of 73 COVID-19 patients hospitalized in China, 39 (53.42%) showed SARS-CoV-2 RNA in stools[49]. Surprisingly, stool samples were positive in 17 (23.29%) patients but respiratory samples were negative and the period of positivity ranged from 1 to 12 d in stools. Xiao *et al*[49] demonstrated that infectious SARS-CoV-2 may be secreted from virus-infected gastrointestinal cells. Therefore, stool samples should be frequently tested using real-time-PCR and transmission-based precautions should be taken into consideration[119].

Furthermore, the probability of SARS-CoV-2 fecal-oral transmission has highlighted the importance of adequate hand hygiene, especially in certain areas. Strict measures must be taken to deal with the stools of COVID-19 patients and the sewage from hospitals. Therefore, patients with preexisting gastrointestinal diseases will be more worried when they are infected with SARS-CoV-2 in addition to potential fecal microbiota transplant donors.

Pharmacologic management of GI symptoms in COVID-19

Microecological preparation: The intestinal flora generates various vitamins, bile acids, immune factors, and fatty acids *via* the decomposition of food and participates in immune system regulation[120]. If there is dysfunction or damage in the intestinal

Table 2 Therapy-specific considerations for inflammatory bowel disease patients

Drug	Effects
Aminosalicylate acid derivatives (5-ASA)	No proof of increased risk of COVID-19 infection
	Continue treatment even in the case of COVID-19 infection
Corticosteroids	Their safety during COVID-19 infection is uncertain
	They can be used at a low dose and for a short period to treat disease relapses
	Discontinue as soon as possible
	Ileo-cecal CD patients can be treated with Budesonide; IUC patients can be treated with Budesonide MMX
Immunomodulators (Thiopurines and Methotrexate)	No proof of increased risk of COVID-19 infection
	Accompanied by increased risk of other viral infection
	Not recommended to start with monotherapy
	Combination therapy with biologics should be maintained
	Recommendations in stopping
	Stable disease
	Sustained reduction in the case of elderly patients and/or significant comorbidities
	Symptom progression of COVID-19 infection
Anti-TNF therapy	No proof of increased risk of COVID-19 infection
	Infusion and dose intervals should be maintained
	Starting with monotherapy (adalimumab or certolizumab)
	Stop in the case of developing symptoms of COVID-19
Anti-IL-12/23p40 therapy (Ustekinumab)	No proof of increased risk of COVID-19 infection
	Monotherapy is recommended
	Stop in the case of developing symptoms of COVID-19
Anti-a4b7 integrin therapy (Vedolizumab)	No proof of increased risk of COVID-19 infection
	Monotherapy is recommended
	Stop in the case of developing symptoms of COVID-19
Janus Kinase inhibitors (tofacitinib)	Although there is no proof of increasing the risk of COVID-19 infection, it may inhibit the immune reaction against viral infections
	Starting is not recommended
	Therapy should be maintained without elevating the dose
	Stop if symptoms of COVID-19 develop

UC: Ulcerative colitis; CD: Crohn's disease; COVID-19: Coronavirus disease 2019; TNF: Tumor necrosis factor; IL: Interleukin.

flora and/or mucosa, the virus may further stimulate infection through this pathway. Other studies have stated that probiotics have the availability to manage diarrhea caused by rotavirus[121]. Also, lactic acid bacteria and Bifidobacteria can contribute to antiviral antibodies in the human body, accelerating virus removal. Therefore, probiotic treatments and/or antiviral drugs, and antibacterial drugs may enhance the symptoms of SARS-CoV-2 diarrhea. Regarding the diagnosis and treatment protocols of COVID-19 in China, the use of intestinal flora regulators is preferred to preserve the intestinal micro-environmental balance and avoid secondary bacterial infection[122].

ACE2 inhibitors: As previously mentioned, ACE2 binds SARS-CoV-2 in cells. Therefore, to avoid SARS-CoV-2 infection the interaction between the receptor connection domain (RBD) of the virus and ACE2 could be blocked. A number of studies have shown that ACE2 inhibitors can regulate bowel metabolism, innate immunity, secretion of antibacterial peptides, and intestinal microbial homeostasis [122]. It was experimentally confirmed that the ACE2 pathway in epithelial cells of the small intestine trigger mTOR *via* nutrient stimulation and/or the tryptophan-nicoti-

namide pathway, thus affecting the composition of intestinal flora, and decreasing GIT indications in mice[60]. Azathioprine is an immunosuppressive agent similar to chloroquine. A recent study evaluated azathioprine and found that it could be regarded as an ACE2 inhibitor[123]. Additionally, *in vitro* studies have shown that Vaccinia virus can be inhibited by azathioprine[124]. With regard to molecular immunity, azathioprine competitively binds to ACE2, using a single chain (ScFv) antibody fragment attached to ACE2 or SARS-CoV-2, *via* the RBD. Thus, the human recombinant ACE2 antibody Fc segment bound by the S protein works by inhibiting attachment of the virus to ACE2[55]. It has been documented that a combination of Chinese medicines (*e.g.*, baicalin in the Chinese medicine Scutellaria baicalensis, scutellarin in Erigeron breviscapus, hesperetin in lime and orange peel, glycyrrhizin in liquorice, and nicotinamide in soybean) and ACE2 resulted in the prevention of SARS CoV-2 infection.

Diet and enteral nutrition: A number of COVID-19 patients have significant anorexia. To ensure effective therapy, the basic energy, absorption, enteral and peristalsis movement of the intestine, and normal function of GIT micro-organisms and mucosal immunity should be taken into account as well as the functioning of the GIT and patients' nutrition[94]. Nutritional risk assessment should be performed in patients with serious COVID-19 and gastrointestinal symptoms[125]. Once the risk of enteral nutrition has been eliminated, enteral nutrition should immediately be restored. If the patient has a gastrointestinal disorder and cannot withstand enteral nutrition, parenteral nutrition should be supplemented with a sufficient supply of energy. Patients in poor condition can be given digestive enzymes[125]. A nasogastric tube can be used for enteral feeding in patients who have undergone mechanical ventilation and are unable to take food orally. A nasal jejunal tube can be inserted when patients are at high risk of reflux aspiration or cannot handle nasogastric tube feeding. Generally, the patient gastrointestinal tolerability should be estimated appropriately and the enteral nutrition program correspondingly adjusted.

LIVER AND COVID-19

Clinical manifestations and pathological changes in hepatic injury in patients with COVID-19

Hepatic injury is a frequent adverse event in both SARS-CoV and Middle East respiratory coronavirus-infected patients and is associated with the severity of disease [126]. A substantial systematic analysis of 11 studies assessing the liver laboratory parameters of 2541 patients with COVID-19 showed increased AST and/or ALT (25%), lactate dehydrogenase (20%), bilirubin (3%) and normal alkaline phosphatase (ALP) [127]. This may indicate minimal direct liver damage caused by ACE2 overexpression in cholangiocytes. A major published study of 5700 patients found that AST and ALT were increased in COVID-19 patients by 58.4% and 39.0%, respectively[128]. Cai *et al* [129] showed that in 41% of patients, gamma-glutamyl transferase (GGT) was increased more than 3 × ULN, while, another research study demonstrated that GGT was raised in severe cases, but without an elevation in ALP[126].

Data from a preprint meta-analysis involving 20 retrospective studies and 3428 COVID-19 patients showed that increased levels of COVID-19 were related to significant increases in the levels of ALT, AST, and bilirubin[130]. Recently, a plethora of studies reported increased serum ALT, AST, and GGT levels in severe compared with mild or non-severe COVID-19 patients[39,131]. A recent meta-analysis associated high marker admission levels with patient mortality[132]. Other studies have shown that the increase in these parameters resulted in a worse pulmonary computed tomography (CT) score[133], increased numbers of ICU patients[12] and longer hospital stays[134]. In patients who died due to COVID-19, the incidence of elevated liver parameters ranged from 58.06% to 78%[32,135].

According to a study conducted by Lei *et al*[132], AST was the first indicator to be considered high when patients were admitted to hospital and was correlated with the highest COVID-19 death rates. A recent study carried out by Guan *et al*[39], around 1100 Chinese patients reported elevated levels of serum AST and ALT of 18% and 26% in non-severe COVID-19 patients, respectively, compared with 56% and 28% in severe COVID-19 patients. Previous findings revealed the crucial role of immune-mediated systemic inflammation in liver dysfunction in patients with serious COVID-19[9]. Recently, Gordon *et al*[136] proposed that mitochondrial proteins may interact directly

with the virus, providing a cause for the high level of AST in the liver profile[136]. Wang *et al*[137] also identified anomalies in liver enzymes, higher radiologic scores as well as a partial pressure differential between alveolar arterial oxygen, higher GGT, disease intensity, higher ferritin, lower CD4+ T and B cells and lower albumin. It is considered that total bilirubin, AST/ALT, and ALT/ALP ratios have helped to predict survival in cirrhotic COVID-19 patients[138]. Serum albumin levels were also markedly lower in patients who died of COVID-19[32]. The adverse path of COVID-19 patients has been shown to include elevated serum levels of IL-6, ferritin, procalcitonin, and C-reactive protein. In addition to reduced albumin content and platelet count, the parallel increase in the level of ferritin, ALT and IL-6 indicates a greater role for liver participation in COVID-19[32,134]. The CT imaging score for pulmonary lesions is known as a hepatic injury indicator. Therefore, patients must undergo careful monitoring of liver function in order to identify any liver insults at an early stage[133]. The incidence of hepatic abnormalities in COVID-19 patients are depicted in Table 3.

Morphological findings regarding the interpretation and description of liver parenchymal changes accompanying COVID-19 are rare, and most are seen at autopsy. It is noteworthy that the first autopsy liver specimen examinations carried out by Xu *et al*[139] showed moderate microvesicular steatosis and both portal activity and mild lobular activity. SARS-CoV-2 infection or drug-related hepatic damage may result in this liver insult. These results matched with those of Liu *et al*[140]. Preliminary research, consisting of 49 patients with COVID-19, showed that the portal intrahepatic system was affected with acute (thrombotic and luminal ectasia) or chronic (fibrous thickening of the vascular wall) features and the intrahepatic blood vessels were abnormally configured. These findings indicated that the major trigger in the pathogenesis of COVID-19 hepatitis is considered endothelial damage or coagulation dysfunction[141]. Additionally, in patients with critical COVID-19 there were no signs of damage to bile ducts or histological changes in liver failure[140]. Several studies failed to identify viral inclusion bodies in liver tissue[139,142]. Furthermore, the study by Li and Xiao[143] demonstrated that cirrhosis and regeneration signs with macrovesicular steatosis and accumulation in hepatocytes, along with atypical lymphocytic infiltration in the portal tract, may occur due to COVID-19. In the portal triad and centrilobular zones, sinusoidal expansion dilatation in zone 3, mild lymphocytic infiltrations and patchy liver necrosis were also revealed.

Moreover, the study by Ramachandran *et al*[144] showed that in hospitalized COVID-19 patients, elevated aminotransferases were associated with higher mechanical oxygen concentrations but did not achieve statistical significance after inflammatory marker measurement. In addition, patients with elevated aminotransferases did not have higher rates of mortality or prolonged length of stay as shown in Table 4.

In China, some patients recovering from extreme COVID-19 were confirmed to have experienced special manifestations including a darkened face and pigmentation during treatment. The key causes of a darkened face and pigmentation were multiple organ injury, in particular liver injury[145,146]. Abnormal hepatic function can easily lead to pigmentation *via* the following three pathways: (1) Liver dysfunction can prevent estrogen from being inactivated[147]. The rise in estrogen decreases thiamine inhibition of tyrosinase *in vivo*, thus increasing the transformation of tyrosine into melanin[148]; (2) Abnormal liver function can cause hypofunction of adrenocortical hormones. The liver does not metabolize melanocytic stimulatory hormone produced by the anterior pituitary gland, which causes greater melanin secretion[149]; and (3) Liver damage can cause bleaching of the face due to iron in the blood that provides the facial skin[150,151] (Figure 4).

Mechanisms of liver injury during COVID-19

Direct damage: It is established that SARS-CoV-2 enters the host cells *via* its binding to ACE2 on the surface of the host cell by the S protein[152,153]. However, the expression of ACE2 levels in liver tissue was estimated to be approximately 0.31% and its expression in bile duct cells was 20 times higher than that in hepatocytes on the basis of single cell sequencing and animal model analysis[154]. Furthermore, elevated levels of γ-glutamyl transferase and alkaline phosphatase were observed in COVID-19 patients[155], and inconsistent with biliary epithelial cells injury, approximately 10% of COVID-19 patients have high levels of total bilirubin. Thus, suggesting that SARS-CoV-2 can bind to cholangiocytes expressing ACE2, resulting in their injury (Figure 5).

Antibody-dependent enhancement: Antibody-dependent enhancement of infection (ADE) may arise in patients with SARS[156]. ADE indicates that the interplay between the virus-based antibody and the CR and/or FC receptor complements increases the

Table 3 Incidence of hepatic abnormalities in patients with severe acute respiratory syndrome coronavirus 2 infection

Ref.	Patient number	ALT (U/L)	AST (U/L)	TB (mg/dL)
Zhou *et al*[32], 2020	191	↑59 (31%)	None	NA
Shu *et al*[227], 2021	545	↑41 (7.5%)	↑35 (10.1%)	↑189 (34.7%)
Huang *et al*[25], 2020	41	NA	↑15 (37%)	NA
Huang *et al*[228], 2020	36	4 (13.3%)	18 (58%)	↑4 (12.9%)
Chen *et al*[26], 2020	99	↑28 (28%)	↑35 (35%)	↑18 (18%)
Ai *et al*[64], 2020	102	↑20 (19.6%)	↑26 (25.5%)	NA
Xu *et al*[27], 2020	62	↑3 (3.75%)	↑3 (3.75%)	NA
Yang *et al*[3], 2020	168	↑9 (8%)	↑18 (17.3%)	↑7 (6.4%)
Wu *et al*[28], 2020	80	↑3 (3.75%)	↑3 (3.75%)	NA
Yao *et al*[229], 2020	40	↑21 (52.5%)	↑16 (40%)	
Xu *et al*[230], 2020	355	↑91 (25.6%)	↑102 (28.7%)	↑10 (25%)
Cai *et al*[231], 2020	298	↑39 (13.1%)	↑25 (8.4%)	↑66 (18.6)
Richardson *et al*[1], 2020	5700	↑2176 (39.0%)	↑3263 (58.4%)	↑24 (8.1%)
				NA
Fan *et al*[1], 2020	40	↑27 (18.2%)	↑32 (21.6%)	↑9 (6.1%)
Guan *et al*[39], 2020	355	↑158 (21.3%)	↑168 (22.2%)	↑76 (10.5%)

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TB: Total bilirubin; NA: Not applicable.

Table 4 Outcome of the patients with severe elevation of aminotransferases in coronavirus disease 2019

Ref.	Outcome of patients	SARS-CoV-2 patients with hypertransaminasemia (*n* = 20)	COVID-19 patients without hypertransaminasemia (*n* = 125)	*P* value
Ramachandran *et al*[169], 2020	Shock	9 (45%)	38 (30.4%)	0.207
	Mechanical ventilation	10 (50%)	30 (24%)	0.028
	Died	10 (50%)	46 (36.8%)	0.324
	Length of stay in days, median (IQR)	7 (4.3, 10.3)	7 (5, 10)	0.78

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; IQR: Inter-quartile; COVID-19: Coronavirus disease 2019.

ability of the virus to reach macrophages, granulocytes, and monocytes (Figure 5). The virus frequently replicates in the aforementioned cells, leading to an increase in virus production and worsening of infection. Previous findings have indicated that SARS-CoV antibodies activate ADE, triggering SARS-CoV in immune cells that do not have ACE2 expression or harm the immune system[157]. Whether ADE can help SARS-CoV-2 infect immune cells through a non-ACE2-dependent pathway and participate in SARS-CoV-2 hepatic injury is an issue of concern.

Systemic inflammatory response syndrome and cytokine storms: Research has revealed that the inflammatory cytokines, including endotoxin ILs, and TNF-α in SARS patients who have liver function impairment have significantly higher levels than patients with normal liver function. Therefore, systemic inflammatory response syndrome (SIRS) and cytokine storms are risk factors for liver impairment in SARS-CoV and in MERS-CoV infected patients[158-160]. Limited pathological findings have indicated that COVID-19 hepatocytes exhibit non-specific inflammatory modifications in patients with serious infection such as Kupffer-cell hyperplasia and moderate proliferation, hepatocyte swelling and steatosis and a limited number of lymphocytes.

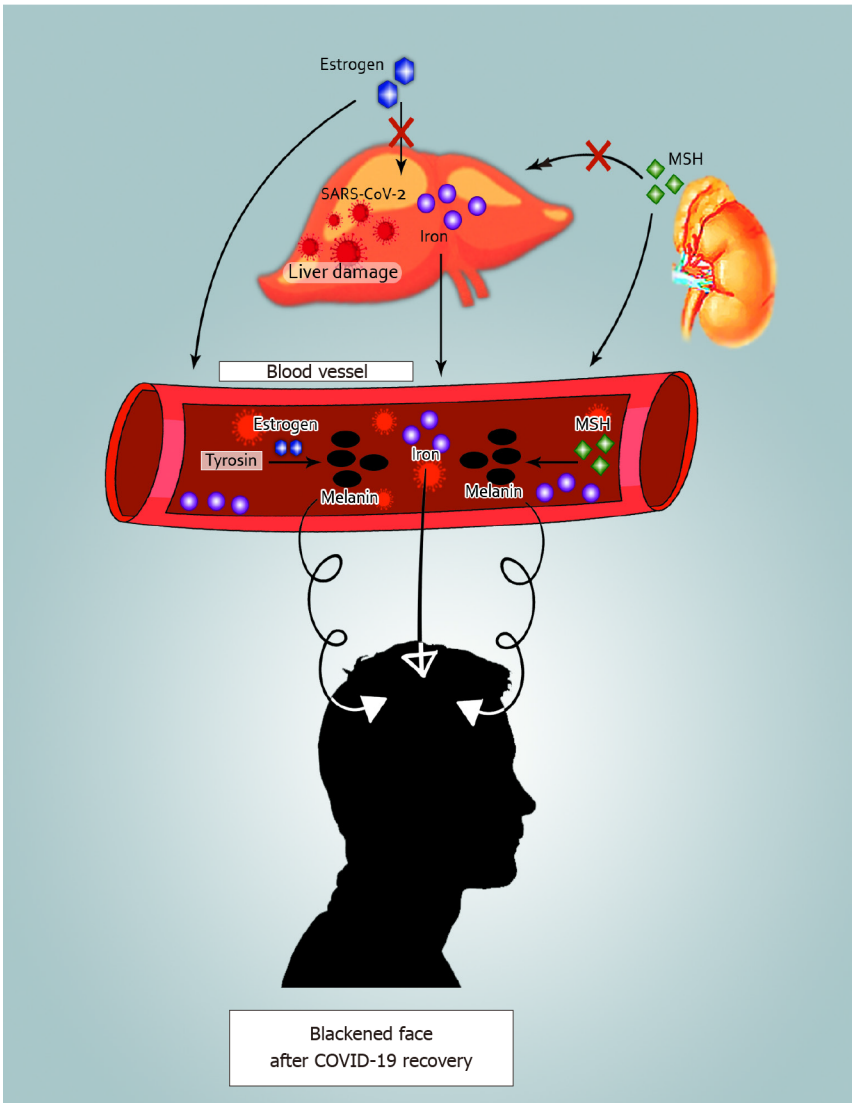


Figure 4 Facial blackness and dull skin after coronavirus disease 2019 recovery. Three possible mechanisms are shown: (1) Iron in the damaged liver drains into blood vessels. Blood with high iron levels can lead to blackening of the face when it supplies the facial skin; (2) Estrogen cannot be metabolized in the damaged liver. Thus, elevated estrogen in the blood enhances the conversion of tyrosine to melanin; and (3) When liver function is impaired, adrenocortical function is hypoactive, and melanocyte-stimulating hormone increases resulting in an elevation in the secretion of melanin. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; MSH: Melanocyte-stimulating hormone; COVID-19: Coronavirus disease 2019.

Furthermore, the amount of IL-2 and IL-6 in COVID-19 serum has been shown to be substantially increased and linked to the seriousness of the disease[4]. Moreover, cytokines secreted by Th1 and Th2 cells in the serum of COVID-19 patients, such as TNF, IL-6, IL-18, IL-4, and IL-10 were significantly intensified in pro-inflammatory cells (CCR4 + CCR6 + Th17)[25,30,139]. Following SARS-CoV-2 infection, a large number of immune cells activate and induce excessive cytokines, such as TNF-α, IFN-μ, IL-6, IL-8, *etc.*, leading to SIRS, acute respiratory distress syndrome, and induction of ischemia, eventually, resulting in cell destruction and necrosis as shown in Figure 4. Not only does such a vicious cycle lead to lung injury but may also progress to multiple organ damage. These results suggest that cytokine inflammatory storms may be one of the essential routes of liver injury (Figure 5).

Ischemia and hypoxia reperfusion injury: COVID-19 patients have various degrees of hypoxia, as more than 40% of cases required oxygen supplementation[30]. Complications such as aspiration and multiple organ failure can cause hypoxia, ischemia and subsequent shock. The suppression of cell survival signal transduction and hepatocyte death may be caused by ischemia and hypoxia, ATP depletion in hepatocytes, lipid accumulation, and glycogen consumption (Figure 5). Furthermore, the respiratory distress syndrome can cause oxidative stress that increases the production of reactive oxygen species (ROS). The ROS and lipid peroxidation products can induce redox-

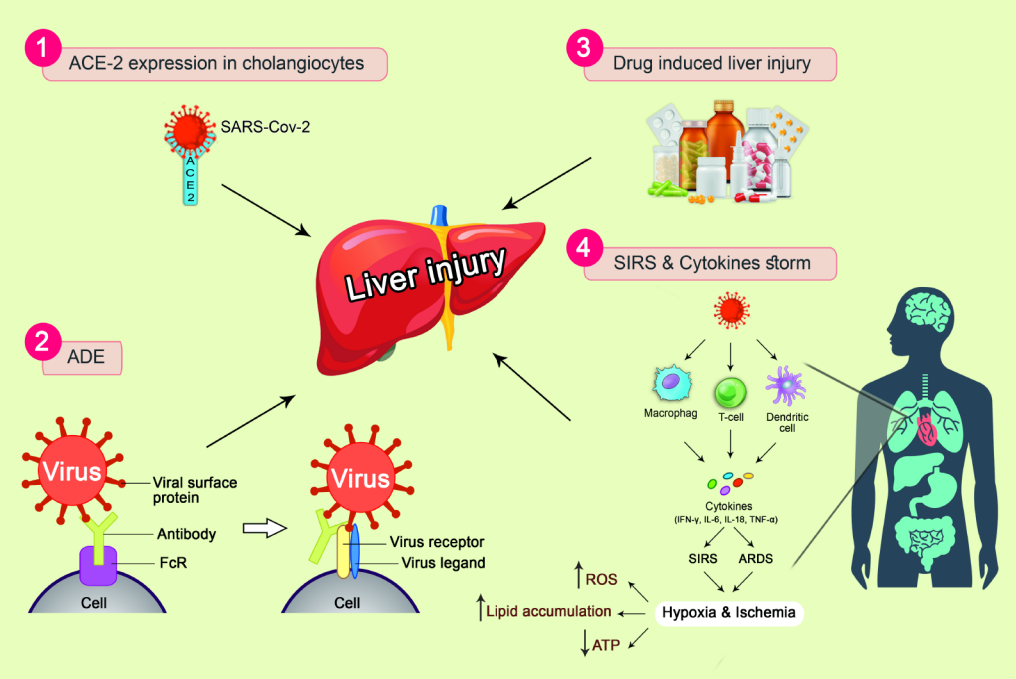


Figure 5 Mechanisms of liver injury in patients with coronavirus disease 2019. (1) Angiotensin-converting enzyme 2-mediated targeting of cholangiocytes; (2) Antibody-dependent enhancement of infection; (3) Systemic inflammatory response syndrome and cytokine storms; and (4) Drug-induced liver injury. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; ACE2: Angiotensin-converting enzyme 2; ADE: Antibody-dependent enhancement of infection; SIRS: Systemic inflammatory response syndrome; ARDS: Acute respiratory distress syndrome; IFN-γ: Interferon-γ; IL-6: Interleukin-6; IL-18: Interleukin-18; TNF-α; Tumor necrosis factor-α; ROS: Reactive oxygen species; ATP: Adenosine triphosphate.

sensitive transcription factors and then release various pro-inflammatory factors leading to liver damage. These changes can exaggerate the ischemia of hepatocytes, influence the excretion of toxic metabolites and eventually stimulate liver injury. Hypoxia is also one of the main causes of liver injury in patients with serious COVID-19[161].

Drug hepatotoxicity: In China, the occurrence of drug-induced hepatic damage including, traditional Chinese patent medicines[162,163], antitumor drugs, antibiotics, antimalarial drugs, and anti-tuberculosis drugs[164,165], is second only to fatty liver disease and viral hepatitis (alcoholic and nonalcoholic). Several COVID-19 patients have a fever and consequently, use antipyretic and analgesic drugs. Therefore, a drug overdose can cause hepatic damage.

Recently, this was observed with abidol, lopinavir, ritonavir and other antiviral medications used to control COVID-19. A recent study published in JCI[166] demonstrated that CAP3A4 plays a critical role of the side-effect and metabolic pathways of ritonavir which can generate electrophilic material, radical free oxygen that can be covalently linked with liver cells leading to lipid membrane peroxidation, membrane integrity disruption of the Ca2+-ATPase membrane, interruption of the internal and external Ca2+ homeostasis of the cells, and finally leading to death. Furthermore, an overdose of the combination of lopinavir and ritonavir can stimulate the hepatic endoplasmic reticulum stress cascade, induce inflammatory reactions, trigger hepatocyte apoptosis through the caspase mechanism, suppress hepatocyte growth, and aggravate liver damage by the production of oxidative stress[167,168]. Some scientists have assumed that SARS-CoV-2 replication can be effectively inhibited by human immunodeficiency virus (HIV) protease inhibitors; although Shen *et al* stated that the risk of liver damage is increased in patients receiving hormones and HIV protease inhibitors[169]. The incidence of liver damage due to different medicines varies. However, the prevalence of liver damage due to more drug types is increasing. The diagnosis of hepatic damage due to medication includes a combination of medical history and appropriate testing to rule out other liver disorders and to estimate the relationship between hepatic injury and suspected medications by causality.

COVID-19 and previously-existing liver disease

Due to the global spread of chronic liver disorders, correlations among patients

presenting with hepatic illness and COVID-19 need to be examined. A preliminary analysis showed that 2%–11% of patients had hepatic disease and COVID-19[170]. In a study of 1099 COVID-19 patients, hepatitis B infection occurred in 23 (2.1%) patients. Serious cases of hepatitis B infection were more likely (2.4% *vs* 0.6%) than milder cases [39]. SARS patients with hepatitis B virus (HBV) or hepatitis C virus (HCV) infection were more vulnerable to serious hepatitis, likely because viral replication was increased during SARS-CoV infection[171]. Older patients with COVID-19 and have other conditions such as diabetes, cardiovascular disease, hypertension, and nonalcoholic fatty liver disease, are more susceptible to liver injury.

Moreover, patients with liver cirrhosis may be more susceptible to infection due to their systemic disease[172], thus, preventing COVID-19 is extremely important. None of 111 patients with COVID-19 in Wuhan had decompensated cirrhosis due to preventive measures. In contrast to this, 17% of 101 patients with COVID-19 developed decompensated cirrhosis at other hospitals where preventive measures were not implemented[173]. Viral transmission could occur during liver transplantation from donor to recipient, as previously described in SARS infection[174]. Recently, Michaels and colleagues showed potential transplantation risks in COVID-19 recipients[175]. The Italian Transplant Authority is carrying out nasopharyngeal swabs to diagnose COVID-19 before donation, with the subsequent exclusion of positive donors[176].

COVID-19 in liver transplant recipients: The worldwide spread of COVID-19 raises additional challenges for organ transplants. Transplant recipients with preoperative decompensated organs and chronic illness tend to contract respiratory viruses. Liver transplant patients, who are exposed to more people, during their wait for transplantation, have an increased risk of cross-infection with COVID-19. Qin *et al*[177] confirmed COVID-19 hepatocellular carcinoma in a patient following liver transplantation. On the 11th day after hepatic transplantation, the patient was diagnosed with COVID-19 following a positive PCR-test, with multiple ground-glass opacities in the left lobes. Long-term immune treatment in transplantation recipients prevents allograft rejection but significantly reduces the ability to protect them against COVID-19 due to their compromised immune system. A systematic analysis of 15 studies involving 223 patients with confirmed COVID-19 showed that simultaneous diarrhea was more likely to be present in patients who had undergone liver transplantation. The higher mortality rates in elderly patients with dyspnea and diabetes were approximately 23% in COVID-19 patients[178]. However, the latest data still do not confirm susceptibility to COVID-19 in liver transplant patients. A number of cases in Italy have indicated that children receiving liver transplants were not at elevated risk of serious lung disease relative to the general population despite being immunocompromised [179]. Similarly, three COVID-19-related deaths observed by D'Antiga[179] at an Italian transplant center were long-term patients on a minimal immunosuppression regimen, rather than recently transplanted patients with complete immunosuppression. Furthermore, a large global observational study by Webb *et al*[180] showed that the risk of death in COVID-19 patients does not substantially increase following liver transplantation.

Pharmacologic management of liver injury in COVID-19

COVID-19 causes mainly transient and indirect liver injuries, which can be caused by hypoxia, systemic inflammatory reactions, and medication. Thus, hepatic damage should be treated by elimination of the basic etiology in the COVID-19 patients. Correction of hypoxia by oxygen supplementation or mechanical ventilation, renal replacement treatment for cytokine storm, and restoration of intravascular effectiveness can enhance liver injuries in the event of septic shock[181]. Also early identification and reduction in the dosage of drugs inducing hepatic damage is crucial. Hepatoprotective anti-inflammatory medicines including L-ornithine-L-aspartate can be used in extreme cases as an adjuvant treatment[182]. It is worth noting that therapeutic drugs may be hepatotoxic, especially in chronic liver disease (CLD) patients. Furthermore, patients treated with immunosuppressive agents should be closely monitored due to drug interactions. Recommendations for the management of CLD, AIH, and immunosuppressed patients during a pandemic are summarized in Tables 5, 6 and 7[183,184].

COVID-19 IN THE ENDOSCOPY UNIT

Although millions of people have remained at their houses to decrease the transmi-

Table 5 Recommendations of the American Association for the Study of Liver Diseases, Asian Pacific Association for the Study of the Liver, and European Association for the Study of the Liver for management of liver disease during coronavirus disease 2019

	Selected recommendations
To limit nosocomial spread	(1) Decrease in-person visits *via* other alternatives such as virtual platforms
	(2) Symptom investigation before entering hospitals to identify COVID-19 patients
	(3) Reduce staffing to essential staff only
	(4) Reduce the frequency of screening and laboratory examinations
	(5) Adhere to recommended PPE by HCW and patients
	(6) Maintain proper social distancing in hospitals
	(7) Postpone unnecessary or elective operations
Management of CLD patients with COVID-19	(1) These patients should be admitted to hospital early
	(2) Prioritization of COVID-19 testing for patients with cirrhosis, CLD patients taking immunosuppressive agents and acute decompensated patients
	(3) Repeated LFTs are advisable
	(4) Early registration in clinical trials as much as possible
	(5) COVID-19 patients with NAFLD should be kept under supervision
	(6) Screening of hepatitis B surface antigen should be taken into consideration
	(7) Drug-induced liver injury should be monitored
	(8) These patients can receive 2-3 g/d of acetaminophen, while limiting the use of NSAIDs when possible
	(9) HBV prophylaxis should be considered before starting immunosuppressive agents
	(10) Stopping Remdesivir in decompensated liver disease patients with ALT more than 5 times the upper limit of normal
Management of chronic viral hepatitis (HCV and HBV)	(1) Despite COVID-19 status, treatment continuity of chronic HCV and HBV is recommended
	(2) In the absence of flare, HBV treatment should be stopped
	(3) For uninfected individuals, HCV and HBV treatment should be continued according to guidelines
Management of HCC	(1) HCC treatment should be continued according to guidelines; however, it can be delayed if necessary
	(2) In the case of COVID-19 patients, delaying elective transplants and resection surgery, and stopping immunotherapy are advisable
	(3) Early admission to hospital is recommended for HCC patients
Management of pre- and post-transplant recipients	(1) Screening donor and recipient for COVID-19 is suggested
	(2) For donors testing positive for COVID-19, transplantation surgery should be postponed
	(3) Prioritization of patients with short-term prognosis
	(4) For post-transplant patients, a reduction in immunosuppressive dose can be considered for moderate COVID-19 cases, while for mild COVID-19 cases, the dose should not be reduced
	(5) For post-transplant recipients, vaccination against pneumonia and influenza is advisable

PPE: Personal protective equipment; HCWs: Healthcare workers; CLD: Chronic liver disease; COVID-19: Coronavirus disease 2019; LFTs: Liver function tests; NAFLD: Nonalcoholic fatty liver disease; NSAIDs: Non-steroidal anti-inflammatory drugs; HBV: Hepatitis B virus; ALT: Alanine aminotransferase; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

ssion of SARS-CoV-2, the risk of COVID-19 amongst health workers is high.

For example, the Chinese National Health Commission determined that approximately 3300 health workers were infected in early March[185]. It is well-known that endoscopic staff are at high risk of airborne droplet infection, conjunctiva contact and surface contact contamination[182,186]. Airway suction and other cough-induced procedures result in an increased risk of SARS-CoV-2 transmission[182]. It must be noted that the possible risk of exposure to feces removal is not confined to upper endoscopy[35].

Table 6 Recommendations of the American Association for the Study of Liver Diseases and European Association for the Study of the Liver for the management of auto-immune hepatitis during coronavirus disease 2019

Selected recommendations	
Virtual platforms are recommended to minimize in-person visits as much as possible	
COVID-19 testing is advised in cases of acute deterioration and liver failure	
Patients with low risk of complications (patients on chronic immunosuppressive therapy)	(1) Frequent patient-provider communications should be closely supervised; (2) Virtual platforms should be used to decrease contact; and (3) Ensure enough drug supply and refills to decrease running out of medications
Patients with moderate risk of complications (symptomatic disease without cirrhosis)	(1) Empiric therapy *via* virtual healthcare platforms as much as possible; and (2) Preventing liver biopsy whenever possible
Patients with high risk of complications (acute flare, decompensated cirrhosis)	(1) Reduce invasive procedures as much as possible; (2) In the case of COVID-19 patients, if lymphopenia develops, dose reduction of antimetabolites is recommended; and (3) In the case of infection, corticosteroids should be attenuated

COVID-19: Coronavirus disease 2019.

Table 7 Recommendations of the American Association for the Study of Liver Diseases and European Association for the Study of the Liver on the use of immunosuppressive therapies in chronic liver disease during coronavirus disease 2019

Suggestions	
(1) Starting with corticosteroids and immunomodulators should proceed; and (2) Risk benefit ratio assessments should be carried out	
Patients on immunosuppressive treatment and not infected with COVID-19	Decreases or adjustment of doses is not advisable
Patients infected with COVID-19 on immunosuppressive drugs	(1) Reduce corticosteroids dose after specialist physician (consider tapering to prevent adrenal insufficiency); and (2) Decreasing the doses of cyclosporine, mycophenolate, and azathioprine is recommended in severe COVID-19 (especially patients with lymphopenia)
Patients requiring initiation of immunosuppressive agents	Starting treatment is suggested in these patients regardless of COVID-19 status

COVID-19: Coronavirus disease 2019.

A prospective study conducted by Johnston *et al*[187], revealed the quantification of unrecognized bacteria to endoscopists exposed to biological samples leading to infection transmission. In this investigation, 227 endoscopic face shields were examined for colony-forming units (CFUs), and 1-15 CFU, 91/227 (40%); 16-30 CFU, 6/227 (2.6%); 30 or more CFU, 6/227 (2.6%) significantly increased after endoscopy. Similarly, in 1999, a research study carried out by Mohandas and Gopalakrishnan[188] in a tertiary care hospital in India of 786 endoscopies (149 lower and 162 therapeutic endoscopies) concluded that the splash rate to the skin of the forearms, feet, and face was 9.5%, while the splash rate to the eyes was 4.1%.

The duodenoscope is the most complex medical equipment that undergoes disinfection after patient use[189]. Virus risk factors include non-compatibility with disinfection guidelines, the promotion of biofilm deposition because of its complicated nature, surface defects and infected automated endoscopes[190,191]. It is considered that endogenous infections due to the gut flora of patients are the most common infections after endoscopy[192]. Exogenous infections such as *Escherichia coli* (71%), *Klebsiella* (14%), and *Enterobacter* (5%) can occur due to contaminated scopes and may be avoided by thorough cleaning[192,193]. Currently, *Pseudomonas aeruginosa* is the most common organism isolated from contaminated endoscopes[194]. Other microorganisms include, *Mycobacteria*, *Helicobacter pylori*, *Clostridium difficile*, HBV and HCV [195]. Recently, studies on duodenoscopy-associated infections including multidrug-resistant organisms, particularly carbapenem-resistant *Enterobacteriaceae*, have surfaced [196]. Numerous infections occurred despite sufficient disinfection, indicating that professional and government bodies should provide additional recommendations for duodenoscope processing[197,198]. To date, The Food and Drug Administration and Centers for Disease Control and Prevention have recommended comprehensive cleaning followed by high-level disinfection for reprocessing of flexible GI endoscopes [199-201].

In COVID-19 patients single-use duodeno-scopes may be important. However, they are not accessible worldwide and have cost-related constraints[202]. Several societies have advocated the use of room negative pressure, particularly for COVID-19 patients, or if endoscopy is urgently needed[203]. Intraprocedural changes such as minimal verbal communication, avoiding procedures in patients with inadequate bowel preparation, and avoiding spillage of GI contents *via* the biopsy channel should be introduced[204]. A previous study reported the use of a "double gauze technique"; one for the endoscopists and the other for technicians in a controlled fashion to prevent the "whip" effect of accessories and spillage of GIT secretions[204] Institutional requirements for minimum staff involved in the procedure have been developed [205]. Thus, the risk of exposure among endoscopy personnel is diminished. Endoscopy techniques performed with moderate sedation without the need for anesthetic agents (endoscopy driven sedation) can also reduce the risk of transmission. However, in the case of procedures requiring general anesthesia, policymakers recently recommended the use of endotracheal intubation to diminish the risk of aerosolization due to suspected or confirmed COVID-19[206]. Other methods to avoid splashes include the use of regular precautions such as full-sleeve gowns and suitable footwear [187].

GAPS IN KNOWLEDGE

Although there is a theoretical risk of fecal-oral transmission of COVID-19, the actual risk of transmission is extremely low. Despite this risk, endoscopy units have been functioning and reopening with no significant outbreaks noted.

CONCLUSION

COVID-19 can lead to lead to acute respiratory infection, and a high rate of morbidity and mortality. The main signs of COVID-19 are respiratory system reactions, and gastro-intestinal symptoms are also very common. COVID-19 patients with GIT symptoms are more probably associated with severe complications such as ARDS and liver damage, with a poor prognosis. Hence, during diagnosis and treatment of the disease, GIT symptoms should be taken into consideration as well as virus transmission *via* the fecal-oral route. In addition, it is advisable to take care of patients with chronic liver disease and they should be treated with medications that are able to prevent inflammatory responses and protect liver functions during COVID-19. Furthermore, the harmful effects of some drugs on the gut and liver during hospitalization must be identified and evaluated frequently. Further studies on the intrinsic relationship between COVID-19, hepatology and gastroenterology are urgently required.

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REVIEW

Management of cholelithiasis with choledocholithiasis: Endoscopic and surgical approaches

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Abstract

Gallstone disease and complications from gallstones are a common clinical problem. The clinical presentation ranges between being asymptomatic and recurrent attacks of biliary pain requiring elective or emergency treatment. Bile duct stones are a frequent condition associated with cholelithiasis. Amidst the total cholecystectomies performed every year for cholelithiasis, the presence of bile duct stones is 5%-15%; another small percentage of these will develop common bile duct stones after intervention. To avoid serious complications that can occur in choledocholithiasis, these stones should be removed. Unfortunately, there is no consensus on the ideal management strategy to perform such. For a long time, a direct open surgical approach to the bile duct was the only unique approach. With the advent of advanced endoscopic, radiologic, and minimally invasive surgical techniques, however, therapeutic choices have increased in number, and the management of this pathological situation has become multidisciplinary. To date, there is agreement on preoperative management and the need to treat cholelithiasis with choledocholithiasis, but a debate still exists on how to cure the two diseases at the same time. In the era of laparoscopy and mini-invasiveness, we can say that therapeutic approaches can be performed in two sessions or in one session. Comparison of these two approaches showed equivalent success rates, postoperative morbidity, stone clearance, mortality, conversion to other procedures, total surgery time, and failure rate, but the one-session treatment is characterized by a shorter hospital stay, and more cost benefits. The aim of this review article is to provide the reader with a general summary of gallbladder stone disease in association with the presence of common bile duct stones by discussing their epidemiology, clinical and diagnostic aspects, and possible treatments and their advantages and limitations.

Key Words: Gallbladder stones; Choledocholithiasis; Laparoscopic cholecystectomy; Rendezvous technique; Management of biliary lithiasis; Endoscopic retrograde cholangio-pancreatography

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Core Tip: Gallstone disease associated with common bile duct stones is a common clinical scenario. Laparoscopic cholecystectomy is considered the gold standard for treatment of symptomatic gallbladder stones; conversely, the best treatment option for bile duct stones remains uncertain and controversial. We discuss herein the epidemiology, natural history, clinical presentations, preoperative diagnosis, management, complications, and different methods of treatment.

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INTRODUCTION

Gallstones are a very common condition among the general population[1]. Generally, this situation does not cause symptoms, but 10%-25% of affected people may have specific symptoms, such as biliary pain and acute cholecystitis, and 1%-2% of these may have major complications[2,3]. In most cases, symptoms and major complications occur due to the migration of stones into the common bile duct (CBD) and this circumstance can cause obstruction of the bile flow in the small intestine, resulting in pain, jaundice, and sometimes cholangitis[4,5]. Primary choledocholithiasis refers to stones formed directly within the biliary tree, while secondary choledocholithiasis refers to stones migrated from the gallbladder. Primary stones are generally brown in colour and composed mainly of calcium bilirubinate; these stones are rare in Western populations and more common in Asia, but the exact aetiology and overall prevalence remain unclear. Secondary choledocholithiasis stone composition parallels that of cholelithiasis, with cholesterol as the most common type[6].

Of the total of cholecystectomies performed every year for cholelithiasis, the presence of CBD stones (CBDSs) is 5%-15%; another small percentage of these will develop CBDS after intervention[7]. The management of CBDSs represents an important clinical problem. In symptomatic patients, the primary goal is to obtain complete clearance of the CBD and cholecystectomy; on the contrary, in asymptomatic patients, there is still no shared diagnostic and therapeutic path[8]. In the last 20 years, the development of new technologies has allowed new diagnostic and therapeutic scenarios, with a consequent critical evaluation of management options. All these have led to a more cautious and patient-tailored preoperative workup based on the patient's risk and ultimately to a multidisciplinary approach[9-11]. However, if on the one hand multidisciplinarity has improved the management of patients with symptomatic cholelithiasis, on the other hand it has shown non-unanimous consent in the choice of treatment for choledocholithiasis: Endoscopic or surgical?

Since the early 1990s, laparoscopic cholecystectomy (LC) has been considered the gold standard of treatment for cholelithiasis[12,13], while endoscopic retrograde cholangio-pancreatography (ERCP) was chosen for isolated CBDSs[5]; no consensus exists to address choledocholithiasis[14,15]. To date, many therapeutic options are available, including laparoscopic, endoscopic, percutaneous, and traditional open techniques, applied both as a combination in a simultaneous way or as a gradual sequence. The most followed therapeutic options are preoperative ERCP followed by LC; LC plus intraoperative laparoscopic CBD exploration (LCBDE); LC plus intraoperative ERCP (rendezvous technique); and, finally, LC plus postoperative ERCP. The preference between one technique and the other is, most of the time, guided by the presence of professional resources and local skills rather than by its verified effectiveness[16-20].

The aim of this review is to provide practical advice and an overview of how to manage patients with cholelithiasis and choledocholithiasis. We take into consideration different diagnostic strategies, as well as the different therapeutic options available.

EPIDEMIOLOGICAL NOTES AND CLINICAL MANIFASTATIONS

Gallstones have a prevalence of 10%-15% in adult Caucasian populations and in the American Indians it can reach up to 70%[1,21]; on the contrary, Asian populations have a very low prevalence[22,23]. The risk factors associated with the most frequent formation of cholesterol stones are female sex, age over 40 years, obesity, and rapid weight loss[24]. The presence of gallstones remains asymptomatic in over 80% of cases, without any complications[21,25]. The risk of developing symptoms or complications varies between 1% and 2.3% per year[2,3,26-29] and in these patients, surgery is necessary.

The most frequent symptom is biliary pain, which is usually constant rather than discontinuous and appears when the gallbladder exit is obstructed by a stone. Most often it occurs in the right upper quadrant but can occur in the epigastrium, retrosternal area, or also the upper left quadrant. This pain has a typical irradiation to the ipsilateral scapula. Pain may be associated with vomiting, and it usually resolves completely but is often associated with other symptoms including flatulence, dyspepsia, and abdominal bloating. If biliary pain persists for more than 12 h, it is very likely that acute cholecystitis is occurring with associated fever, tachycardia, and then systemic inflammation.

Among the classic complications of gallbladder stones, we must remember acute cholecystitis which can resolve spontaneously or result in mucocoele, empyema, gangrenous cholecystitis, and emphysematous cholecystitis. Another complication is chronic cholecystitis, which usually occurs after recurrent episodes of biliary pain associated with phases of acute inflammation that result in fibrosis. Less frequent but not uncommon are Mirizzi syndrome, cholecysto-enteric fistulas, and the risk of malignant transformation in association with chronic cholecystitis. Migration of stones from the gallbladder to the CBD facilitated by gallbladder contractions can also be listed as a complication of gallstones. In the CBD, they can reach the duodenum following the bile flow or they can remain in the choledochus. In the latter case, they can remain asymptomatic or cause a variety of biliary problems. Incidence of choledocholithiasis increases with age, and between 20% and 25% of these patients with symptomatic gallstones have stones in the CBD and in the gallbladder[7,8]. The Swedish registry[30] showed a prevalence of 11.6% of choledocholithiasis detected during intraoperative cholangiography in patients with symptomatic gallbladder stones. Important studies concerning the prevalence of choledocholithiasis in patients with asymptomatic cholelithiasis are currently lacking.

Complications due to the presence of stones of the CBD are biliary pain and obstructive jaundice secondary to obstruction and subsequent dilation of the biliary tree[31], cholangitis due to bacterial infection facilitated by biliary obstruction, and ultimately acute biliary pancreatitis, which together with pain represent the largest percentage of such. Complications that most require endoscopic or surgical intervention are those that occur in the presence of choledocholithiasis.

PREOPERATIVE INVESTIGATIONS

The main issue for the correct management of cholecysto-choledocholithiasis is to arrive at the diagnosis by reducing the useless and invasive diagnostic procedures that are often performed inappropriately following non-linear clinical hypotheses. Generally, suspicion of the disease arises from the presence of specific symptoms associated with an alteration of the cholestasis indexes followed by an abdominal ultrasound (US) examination. Signs and symptoms of these are well known and previously mentioned. Instead, it is necessary to focus on preoperative serological and instrumental diagnostic investigations.

Serology

Normal values of liver function tests are considered predictive of the absence of CBDS. Yang *et al*[32] in 2008 presented a study conducted on 1002 patients undergoing LC, which confirmed the absence of CBDSs in the presence of normal values of liver function indexes. Total and direct bilirubin and gamma-glutamyltransferase demonstrated the highest sensitivities at 94.7% and 97.9%, respectively. Gurusamy *et al* [33] in 2015 proposed a systematic review on the diagnostic accuracy of liver function tests, concluding that the sensitivities of bilirubin and alkaline phosphatase were 84% and 91%, respectively, while the corresponding specificities were 91% and 79%.

Therefore, an alteration of total and direct bilirubin, gamma-glutamyltransferase, and alkaline phosphatase[34,35] has always been associated with the suspicion of choledocholithiasis, as well as the presence of elevated hepatic cytolysis values, as it was found in the presence of unrecognized CBDSs[34,36,37]. To date, total and direct bilirubin are considered the most reliable markers for the suspicion of choledocholithiasis, but the other liver biochemical tests should not be underestimated[38].

Radiological examinations

Diagnostic suspicion is essential to select patients to be directed to a more accurate study. Several predictive models have been developed but the association between the presented symptoms, biochemical data, and an US examination of the abdomen is often the most suitable way to identify high risk patients[35,39-42]. Abdominal US is the first non-invasive, inexpensive, and practicable examination to be performed almost anywhere[38,43,44]. This examination is also useful for evaluating the presence of other liver diseases. The high sensitivity of US (96%)[45] for the study of the gallbladder and the identification of stones inside it is due to the proximity of the organ to the abdominal wall and the absence of interposed gas.

Unfortunately, US often fails to confirm the presence of CBDSs because they do not show the characteristic acoustic shading or are located in the distal part of the choledochus, where they can be obscured by gas[46]. In these situations, it is necessary to evaluate the presence of indirect signs of obstruction, such as dilation of the CBD. Even the definition of the concept of dilation of the CBD is not very clear, the most used range is between 5 mm and 11 mm[47-49], bearing in mind that some dilation can also occur after cholecystectomy and with advancing age. Another indirect sign of suspected choledocholithiasis can be provided by the number and size of gallbladder stones[44,50]. US confirmation of small gallstones increases the possibility that they can migrate into the CBD[51,52]. Wilcox *et al*[53] in 2014 and Qiu *et al*[54] in 2015 reported two studies that had demonstrated, with different techniques and percentages, the presence of CBDSs in patients who had normal findings on liver function tests and US. For these reasons, abdominal US is certainly to be performed in the first instance, but in the suspicion of choledocholithiasis even with a normal serology and US evaluation it is a good rule to deepen the diagnosis with second-line examinations, which are more expensive and invasive.

Computed tomography (CT) is often done in emergency situations and for abdominal pain assessment. It is considered a second-line examination for the diagnosis of this pathological condition, but the literature expresses heterogeneous judgments regarding its diagnostic value and it is usually considered a non-definitive test. In fact, many gallstones are similar in density to the surrounding bile and lack of calcium; these occurrences limit the visibility of CT and therefore its sensitivity. The CT scan increases exposure of patients to X-rays and leads to higher costs than US. It is considered more accurate than the latter in the identification of CBDSs[55] but certainly inferior to magnetic resonance cholangiography (MRC)[56]. Greater diagnostic precision has been achieved with helical CT[38,57] and CT cholangiography [56,58]. CT cholangiography has shown a sensitivity in some studies and a specificity almost comparable to that of MRC[56,58]. The new multi-detector CT has shown a sensitivity of 78% in the portal venous phase and a specificity of 96% in the identification of CBDSs[57]. On the other hand, Kim *et al*[59] in 2013 reported that in 17% of cases, the 64-detector CT does not visualize CBDSs, due to size of < 5 mm, black/brown colour, and the patient's age. The greater radiological exposure and lower diagnostic power of CT compared to MRC make the latter examination preferable. However, CT examination has reduced execution times and extreme availability and diffusion, and these aspects could in the future assign it a new role in this field.

MRC and endoscopic US (EUS) are currently the radiological examinations used routinely to avoid unnecessary and more invasive procedures, such as ERCP. Specifically, MRC is now considered the most accurate non-invasive procedure for the detection of CBDS, with a sensitivity of 93% and specificity 96%[60]. It is non-invasive, does not require contrast, and can be performed without anaesthesia.

Meeralam *et al*[61] in 2017 demonstrated that diagnostic accuracy of EUS and MRC was high for both methods: Sensitivity of 97% *vs* 90% and specificity of 87% *vs* 92%. This was mainly due to the significantly higher sensitivity of EUS in the identification of small stones, while the specificity was not significantly different. The major limitation is the cost, and routine MRC for biliary disease without positive laboratory tests for CBDSs is not cost-effective[62]. Other disadvantages of MRC are its suboptimal availability in non-tertiary care centres and non-therapeutic purposes of the technique; in fact, in the case of CBDS diagnosis, it will be necessary to use other

treatment procedures. Its use is difficult in patients with severe obesity and claustrophobia, and in the presence of metallic foreign bodies or other metallic devices. Small stone diameter (< 5 mm) and peripancreatic oedema have been shown to reduce the accuracy of CBDS identification[63]. Regardless of the overall effectiveness, the points in favour of this diagnostic procedure are its non-invasiveness and higher spatial and three-dimensional resolution, with the use of dedicated software, compared to EUS; in any case, it remains an operator-dependent diagnostic investigation. However, despite some of its limitations and low territorial diffusion, it should always be preferred before referring patients to other more invasive procedures.

Percutaneous transhepatic cholangiography (commonly referred to as PTC) is a procedure that is performed with percutaneous cannulation of the intrahepatic biliary system, with contrast injection monitored by fluoroscopy. This technique can demonstrate biliary anatomy, including the size, number, and position of the stones, in the same way as ERCP. PTC is rarely used for the diagnosis of choledocholithiasis following the advent of modern radiological and endoscopic imaging techniques. However, it can be the initial component of the percutaneous transhepatic therapies for biliary tract disease, including choledocholithiasis, often when ERCP is not feasible.

Endoscopic examinations

All endoscopic procedures are invasive for the patient. They are also diagnostic and therapeutic procedures. ERCP involves cannulation of the ampulla of Vater and then of the CBD; through the injection of contrast medium under fluoroscopy, defects in filling are observed. This method is often used as a procedure of choice for evaluating the presence of choledocholithiasis, but complications can occur in as many as 8% to 12% of patients, usually manifesting as pancreatitis[64-66]. Due to its invasiveness and possible complications, ERCP is recommended for patients with a high probability of choledocholithiasis; this endoscopic examination conducted by expert operators can also be an adequate treatment[38]. Although most endoscopists routinely reach the second portion of the duodenum, there are some situations that can make this manoeuvre difficult. Sometimes, the papilla major is difficult to identify and cannulate; this then represents a time of stress and danger for the operator as well as for the patient, such as when the cannula is placed in a duodenal diverticulum[67]. Previous surgical procedures on the stomach are another frequent cause of ERCP failure. The second duodenal portion is difficult to reach after a Roux-en-Y reconstruction, omega anastomosis, and gastric by-pass, and after gastrectomy with duodenal stump closure and Billroth II reconstruction[68,69]. In those cases, diagnosis and treatment must be conducted surgically.

In the past decades, ERCP has been widely used for the diagnosis of CBDS; today, this procedure is being abandoned, especially in those patients who have a low or moderate risk of disease[70]. ERCP accuracy is lower than that of EUS and MRC, especially in cases of dilation of the CBDS and presence of small stones[71]. Furthermore, this procedure has a non-negligible morbidity and exposes the patient to X-rays and complications such as pancreatitis. The use of sphincterotomy during ERCP is therapeutic and mandatory in high-risk patients, but this procedure in untrained hands can increase morbidity and mortality, possibly causing duodenal perforation or haemobilia. Therefore, the use of ERCP is recommended in those patients with strong suspicion of choledocholiasis; in other cases, the use of EUS or MRC is preferable[38]. The increase of the laparoscopic approach for the treatment of patients with cholelithiasis, which has completely replaced open surgery, has revived the therapeutic role of ERCP. To date, for the management and treatment of cholecysto-choledocholithiasis, the most used approaches are in two sessions or in a single session, and in both cases the ERCP retains a fundamental role.

A further refinement of the classic endoscopic procedures is represented by the endoscopic US, which uses an US probe mounted on the tip of an endoscope. EUS does not use ionizing radiation. In comparison to ERCP, it is more useful for stones smaller than 5 mm and has a complication rate between 0.1% and 0.3%[38]. EUS may not be adequate in patients undergoing gastric surgery, as the anatomical situation would be altered. Like transabdominal US, EUS is not limited by bowel gas, but it is always an operator-dependent procedure. Giljaca *et al*[72] in 2015 published the results of a systematic review, reporting high rates of diagnostic accuracy for both EUS and MRC for choledocholithiasis. The authors found a sensitivity of 95% and specificity of 97% for EUS, and a sensitivity of 93% and specificity of 96% for MRC. The choice between EUS and MRCP, for intermediate probability choledocholithiasis, is based on the resource availability, personal experience, and costs[73].

INTRAOPERATIVE MANAGEMENT

LC is confirmed as the gold standard for the treatment of symptomatic cholelithiasis [12,13], but the therapeutic choice for CBDS is not clear. For the latter situation, the available strategies, conducted in a minimally invasive way, can be divided into two-session treatments and single-session treatments. The first category includes preoperative ERCP followed by LC, and LC followed by postoperative ERCP; the second category includes LC with LCBDE, and LC with intraoperative ERCP, also called the rendezvous technique.

Two-session options

Preoperative ERCP followed by LC is the most frequently used treatment worldwide [74]. Various studies have shown that this two-session approach is safe and effective [17,75,76]. Its limits are represented by a percentage between 40% and 70% of negative results that expose patients to unnecessary and risky endoscopic manoeuvres[77-79]. The development of diagnostic techniques such as MRC and EUS have increased the anatomical visualization of the biliary tract and have showed a high sensitivity and specificity for preoperative diagnosis of CBDS, all without the aid of special instruments and with less invasive approaches[80,81]. Lefemine *et al*[82] demonstrated that more than 50% of patients with CBDS have spontaneous passage of the stones, and 12.9% of patients already undergoing ERCP still had CBDSs during LC[83]. These events could be due to an ineffective ERCP or to a further migration of stones during the latency time between endoscopic and surgical intervention. Preoperative ERCP could increase the conversion rate from LC to open surgery, prolong the operating times, and have higher morbidity. It should also be emphasized that the high morbidity mainly translates into postoperative infections and consequently a longer hospital stay[84-86]. Preoperative ERCP followed by LC often requires two rounds of anaesthesia and two hospitalizations, and in the time between the two procedures, some patients may escape LC, being satisfied by the results of the preoperative ERCP [87,88]. Therefore, for this treatment modality, it is a good rule not to delay the LC too much and also to avoid the occurrence of recurrent events[89].

Another option is LC followed later by postoperative ERCP. This technique is rarely performed as the first choice for the treatment of cholelithiasis with choledocholithiasis because in a low percentage of cases it fails the intended purpose. The failure may be due to the operator's lack of experience, the absence of a guide wire, and a site altered by the previous surgery for inflammatory phenomena. The negative result of the endoscopic treatment necessarily leads to surgery, which can be more demanding than if it had been performed before. The postoperative ERCP retains a role when the intraoperative laparoscopic exploration fails or if during a LC, the presence of CBDS is found with the impossibility of performing it intraoperatively. Obviously, also in this case, the patient is subjected to two rounds of anaesthesia, with possible increase in morbidity, lengthening of the hospital stay, and higher costs[90,91].

One-session options

One-session procedures have confirmed benefits now, both in suspected cases and in proven cases of CBDS. These procedures are believed to be efficient, safe, convenient, and well accepted by patients, as the two different pathological conditions are resolved in a single surgery with single anaesthesia[92,93]. Regarding laparoscopic exploration of the CBD (Figure 1), some authors have described stone removal rates ranging from 94% to 98%, low morbidity, and a mortality rate of 0%[94,95]. Through laparoscopic exploration, negative events that can occur during endoscopic sphincterotomy, such as pancreatitis, perforation, bleeding, cholangitis, and malignancies of the CBD, are avoided. However, this technique has some drawbacks.

First, the procedure requires high-level skills in laparoscopic surgery with long learning curves and there is a need for dedicated instruments; it is understandable that these qualities may not be present in all treatment centres[96]. Many surgeons prefer the transcystic approach, which is considered less invasive and less complicated; however, choledochotomy is recommended in cases of dilated CBD, large diameter or multiple stones, impacted stones, and stones with intrahepatic localization[97-99]. The transcystic exploration route is often more challenging, due to a small diameter and tortuous and low-base implant duct. We can affirm that it is logical to start transcystically but in case of difficulty to move on to exploration through choledochotomy[100,101]. During laparoscopic exploration, stone removal can be guided fluoroscopically or by choledochoscopy. The use of a flexible choledochoscope is the most preferred because it increases precision and is under direct visual control. The

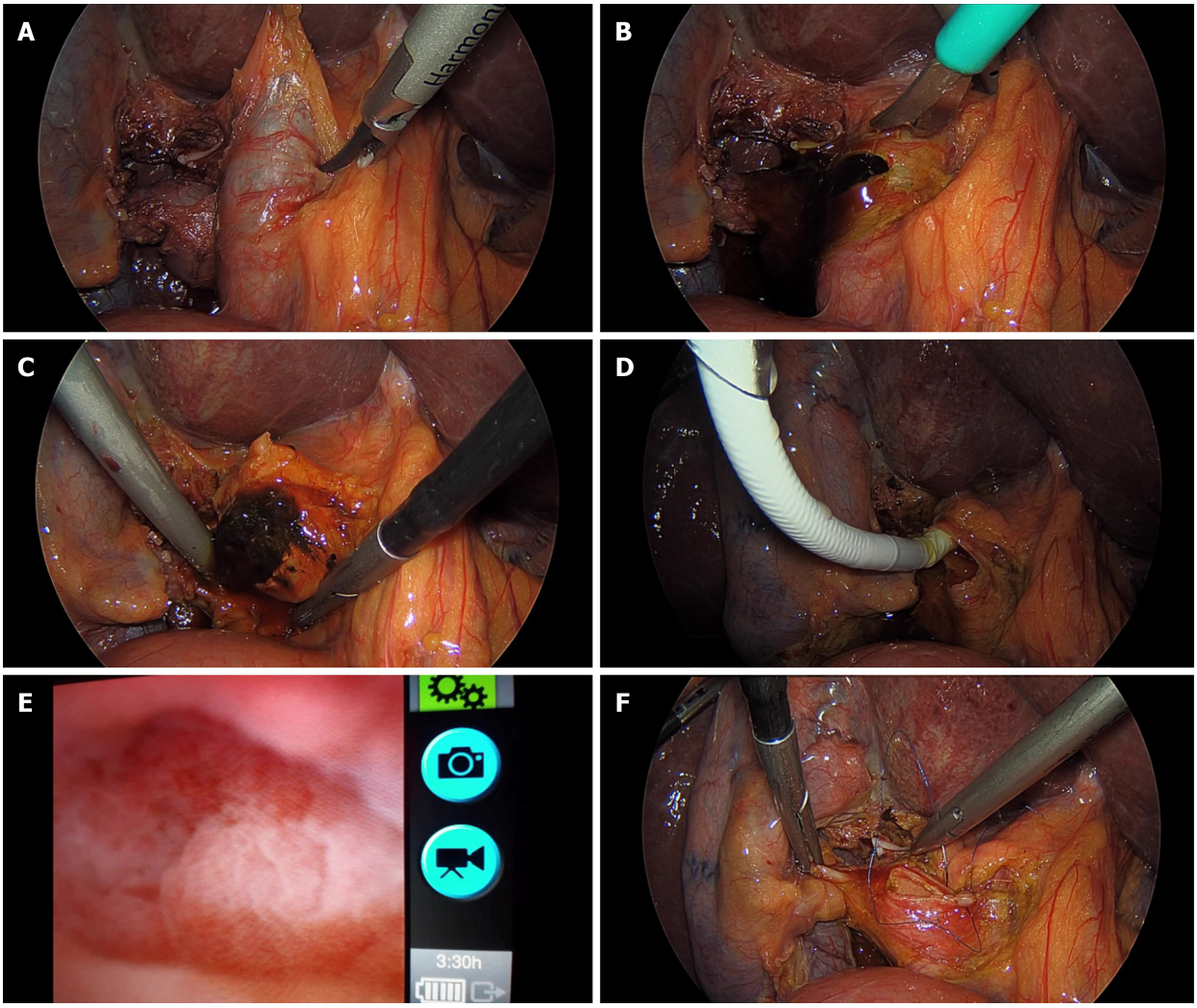


Figure 1 Intraoperative images: Laparoscopic exploration of the common bile duct. A: Common bile duct (CBD) dilation; B: CBD section; C: Stone extraction; D: Insertion of the choledochoscope into the CBD; E: Choledochoscopic image of the CBD; F: Suture of CBD.

choledochoscope also presents some criticalities; first of all, it is a fragile instrument that can break during the procedure, thereafter needing a double monitor for laparoscopic and choledochoscopic viewing and causing an increase in costs. Fluoroscopic guidance exposes the patient to radiation, increases the length of the procedure, and requires instrumentation that can hinder the operator's movements. In this regard, Topal *et al*[102] in 2007 compared the two procedures; the results showed a difference in the duration of the surgical time, being shorter in the group of patients treated with a flexible choledochoscope.

After the choledochotomy, the closure of the CBD can be performed directly or with the positioning of the T-tube endoprosthesis[103,104]. The T-tube provides easy percutaneous access for cholangiography and extraction of preserved stones[105]. However, the T-tube can accidentally detach, promoting CBD obstruction, bile loss, persistent biliary fistulas and skin abrasions, cholangitis from exogenous sources through the T-tube, and dehydration and salt depletion[106-109]. The T-tube also requires long and continuous management, limiting the patient's quality of life[110]. Some authors have concluded that primary closure is preferred over T-tube placement, as the latter increases operative time and hospital stay[111]. The most common complications of this technique are CBD tearing, leakage of bile, stitched T-tubes, and the formation of strictures[99]. The results of three recent studies did not show biliary strictures, and approximately 640 patients were observed undergoing LCBDE with an average follow-up of more than 3 years[112-114]. LCBDE for the treatment of primary choledocholithiasis showed a high rate of stone recurrence (36.4%-41.7%)[115,116], possibly because it does not affect the biliary tract structure and lithogenic environment[117]. Endoscopic and surgical techniques for extracting these stones are equally valid in terms of efficacy, morbidity, or mortality[5]. However, endoscopic sphincterotomy may leave a higher incidence of retained stones (16%) compared to the

surgical approach (6%)[31]. Currently, endoscopic sphincterotomy or endoscopic papillary balloon dilation represent the first-line treatment for initial primary choledocholithiasis, while the LCBDE should be performed for large stones, keeping the sphincter of Oddi intact.

Another single-session minimally invasive procedure for the treatment of cholecysto-choledocholithiasis is ERCP during the LC, also called the rendezvous technique (Figure 2). This approach was found by many experts to be safe and effective[77,118-120], and to require single hospitalization and single anaesthesia. For the exploration and extraction of the stones, the CBD is not opened, postoperative ERCP is avoided, and in case of extraction failure, surgical exploration is carried out during the same operation, with cost reduction. Despite these benefits, the procedure is not widespread, probably because operative endoscopy is not present in all treatment centres, and often when this exists, there is no expert operator. Intraoperative ERCP can be conducted in several ways. One of these is that after catheterization of the cystic duct, cholangiography is performed and in the event of a positive result, ERCP is performed and subsequently the LC is completed[121]. Some authors have proposed performing LC as a first step and then intraoperative ERCP[122]. Cavina *et al*[123] in 1998 proposed the rendezvous technique, a combined laparo-endoscopic approach which, due to its simplicity, has been widely used as a single-session treatment. A guide wire is introduced through the cystic duct and out the ampulla of Vater into the duodenum. Using side-viewing duodenoscope, the protruding guide wire is grasped by a snare or basket and a standard sphincterotome is threaded over it to facilitate endoscopic sphincterotomy and/or stone removal.

Other authors have proposed further variations to the rendezvous technique[124]. In one, after the endoscopic sphincterotome is passed over the guide wire through the papilla, an endoscopic sphincterotomy is performed under the direct vision of a simultaneously positioned duodenoscope. The rendezvous technique compared to classical ERCP has shown advantages in cannulation in the supine position and lower incidence of postprocedural pancreatitis[125]. The explanation for these results is that during classical ERCP, contrast medium is injected and often inadvertently cannulated into the pancreatic duct[126,127]. However, problems can occur during the rendezvous technique. The insertion of the guide wire may not be easy, due to the valves present in the cystic duct; inside the CBD, it can twist, due to the presence of impacted stones, and can not enter the papilla. During the ERCP, due to the insufflation of air, the bowel can stretch and cause difficulties for the LC. In expert hands, these obstacles can be overcome; the use of an atraumatic laparoscope has been proposed for a clamp positioned on the first jejunal loop or a bowel desufflator[128,129], as well as to completely dissect the Calot triangle and the attachment between the liver and gallbladder or to even remove the gallbladder from the liver bed until the last centimetres are left at the fundus before the starting of the endoscopic phase[130].

There are no definitive indications and contraindications in the literature for two-session or one-session treatment. It is clear that, in those centres that have technical and human resources available, all patients with cholelithiasis and concomitant choledocholithiasis can be treated *via* a one-session approach. La Greca *et al*[131] in 2008, after observing 80 consecutive patients affected by cholecystolithiasis and diagnosed or suspected CBDSs treated by the rendezvous technique, evaluated the factors that suggest the preference of the rendezvous technique over other treatments (laparoscopic CBD exploration and sequential ERCP-endoscopic sphincterotomy, commonly referred to as ERCP-ES) (Table 1)[131]. On the other end, the advantages and disadvantages of one-session compared to two-session are clearer. The first procedure is associated with a shorter hospital stay, fewer procedures, and less cost, but local resources and expertise must be present. Other factors that can influence its choice are patient fitness, clinical presentation, timing of CBDS diagnosis (preoperative or intraoperative), and the surgical pathology. Finally, patients with acute biliary pancreatitis and septic shock from cholangitis are not ideal candidates for the one-session option, and could benefit from two-session[132,133].

HINTS ON SUPPLEMENTARY TREATMENTS

Through an endoscopic biliary sphincterotomy or a laparoscopic procedure, the extraction of ductal stones may not be completed or be difficult; in these cases, the open surgical approach still retains an important role, like other additional techniques.

Table 1 Factors that suggest the preference of the rendezvous technique over other treatments

Main indications for the laparo-endoscopic RV	RV preferable *vs* laparoscopic CBD exploration	RV preferable *vs* sequential ERCP-ES
(1) CBD stones not easily extractable	(A) Need of higher surgical skill	(a) Risk of synchronization
through the cystic duct	(B) Longer operation time	(b) Risk of unnecessary ERCP
Positive factor - > (time reduction)	(C) Need of biliary drain	(c) Risk of difficult retrograde
		cannulation
(2) Multiple small CBD stones and large friable stones	A, B, C +	a, b, c
Positive factor - > (reduction of risk of recurrence)	(D) High risk of residual fragments and recurrence	
(3) Any type of CBD stones with delayed passage of the	A, B, C, D +	a, b, c
contrast medium during IOC or T-tube-IOC after	(E) High risk of undertreatment of chronic	
laparoscopic CBD exploration	papillitis and of maintenance of underlying causes	
Positive factor - > (reduction of risk of recurrence)		
(4) CBD stones with previous cholangitis	A, B, C, D +	a, b, c +
Positive factor - > (reduction of risk of recurrence)	(E) High risk of maintenance of underlying causes	(d) Avoidance of contrast medium injection
	at the papilla	with risk of recurrence of cholangitis
(5) CBD stones after recurrent acute biliary pancreatitis	A, B, C, D, E	a, b, c, d +
or hyperbilirubinemia		(e) risk of recurrence of ERCP
Positive factor - > (iatrogenic risk reduction)		related acute pancreatitis
(6) Known or unsuspected sphincter of Oddi dysfunction,	A, B, C, D, E	a, b, c, d, e
cholecysto-lithiasis with or without CBD stones		
Positive factor - > (iatrogenic risk reduction)		
(7) CBD stones and/or abovementioned problems in patients	A, B, C, D, E +	a, b, c, d, e +
with Billroth II during open cholecystectomy	(F) Manual drive of the endoscope by the surgeon	(f) more difficult ERCP
Positive factor - > (iatrogenic risk reduction)	in the afferent jejunal loop	
(8) CBD stones, SOD, acute pancreatitis in children/CBD	A, B, C, D, E +	a, b, c, d, e, f +
stones in patients with normal or thin CBD	(G) difficult laparoscopic CBD exploration and risk	(h) avoidance of
Positive factor -> (iatrogenic risk reduction)	of stenosis of the suture	sphincterotomy in children
(9) CBD stones and/or SOD after failure of preoperative	A, B, C, D, E	a, b, c, d, e, f
ERCP-ES or recurrence of acute biliary pancreatitis		
Positive factor - > (iatrogenic risk reduction)		
(10) Inexperienced surgeon for laparoscopic CBD exploration	A, B, C, D, E, G	a, b, c, d, e, f
Positive factor - > (iatrogenic risk reduction)		

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Open surgical procedure

Four decades ago, cholelithiasis was treated exclusively by open cholecystectomy and similarly, choledocholithiasis was managed by open CBD exploration, which was performed by duodenotomy and sphincterotomy or bilioenteric anastomosis[134]. Open surgery is now considered obsolete, but the recent literature has shown its superiority over ERCP in the clearance of CBDS, with lower morbidity and mortality rates (20% *vs* 19% and 1% *vs* 3%, respectively)[31,135]. Open exploration of the CBD can be conducted through a coledochoenterostomy or a sphincterotomy; the choice depends on the surgeon's experience[136]. Some authors prefer coledochoenterostomy for CBD with a diameter greater than 2 cm, in order to create a large opening between the bile duct and intestine. An emerging problem is that open biliary surgery is performed increasingly less outside specialized centres in hepato-bilio-pancreatic surgery; this raises new questions regarding the most appropriate management of those patients. Their number is scarce but not negligible, and their complex cases often need conversion or revision using an open approach by skilled surgeons.

During the sphincterotomy, an incision of approximately 1 cm is made in the distal part of the sphincter musculature. A catheter or dilator is passed distally and a Kocher manoeuvre is performed, followed by duodenotomy at the level of the ampulla. The dilator exposes the ampulla in the operating field, where it is sufficiently incised along its anterosuperior border with subsequent removal of the impacted stone[137]. Choledocoenterostomy is commonly performed as a side-to-side choledochoduodenostomy for dilated CBD with multiple stones. These patients require drainage for good long-term results without recurrence of jaundice or cholangitis[138]. The most used technique is that of a side-to-side hand-sutured anastomosis between the supraduodenal CBD and the duodenum[139]. Kocher's manoeuvre is performed to expose the distal CBD. Choledochotomy is performed for 2-3 cm to the lateral border of the duodenum. Anastomosis is performed with interrupted absorbable sutures. The biggest complication that can occur is sump syndrome caused by food or other debris trapped in the distal part of CBD[140]; its management is endoscopic with ERCP/ES. Another option may be choledochidjejunostomy with a roux-en-Y loop, but performed by expert hands.

Lithotripsy

Lithotripsy could be considered the ideal management of CBDS, as it resolves the disease without interruption of the CBD wall and without performing a sphincterotomy. However, this technique cannot be considered definitive in the treatment of cholecysto-choledocholithiasis since the genesis of the stones is secondary to the lithogenic bile in the gallbladder, and for this reason the recurrence rate would be very high. Fragmentation of gallbladder stones would increase the percentage of their migration into the choledochus. Another advantage of this technique is certainly that of being performed in a single application. Lithotripsy is not able to avoid cholecystectomy but it can be a valid therapeutic alternative in those patients already cholecystectomized or for whom it is not indicated. However, it cannot be overlooked that lithotripsy requires dedicated instrumentation and skilled personnel, which are not always available, thus limiting its diffusion. There are various approaches described for stone fragmentation: Mechanical, electrohydraulic, laser, and extracorporeal shock wave[141-143].

Endoscopic mechanical lithotripsy is usually performed after failed endoscopic sphincterotomy for CBDS through a Dormia basket or balloon catheter. CBD clearance is reached in about 80%-90% of cases[143]. Failure may be due to CBDS size exceeding 3 cm, since stones could not be captured, and stone impaction in the CBD[144].

Endoscopic electrohydraulic lithotripsy may be used in cases of difficult CBDS. A large operating channel accommodates a 4.5 Fr calibre probe with an electrohydraulic shock wave generator sending high-frequency hydraulic pressure waves. In order not to cause damage to the surrounding tissue, the probe must be positioned as close as possible to the stone. The stone removal rate ranges from 74% to 98%[145,146]. This procedure requires costly and fragile instrumentation and good coordination of two skilled endoscopists.

Endoscopic laser lithotripsy has been used to fragment large stones under fluoroscopic visualization, because of the risk of heat-induced biliary damage[147]. Today, single-operator steerable cholangioscopy allows the safer use of laser lithotripsy with direct vision[148]. A removal rate of CBDS has been reported of about 93%-97%, with a complications rate of 4%-13%[149,150]. The holmium laser is the newest one, but its use is very expensive[151]. Laser lithotripsy has recently been proposed with laparoscopic, open surgery, or percutaneously approaches.

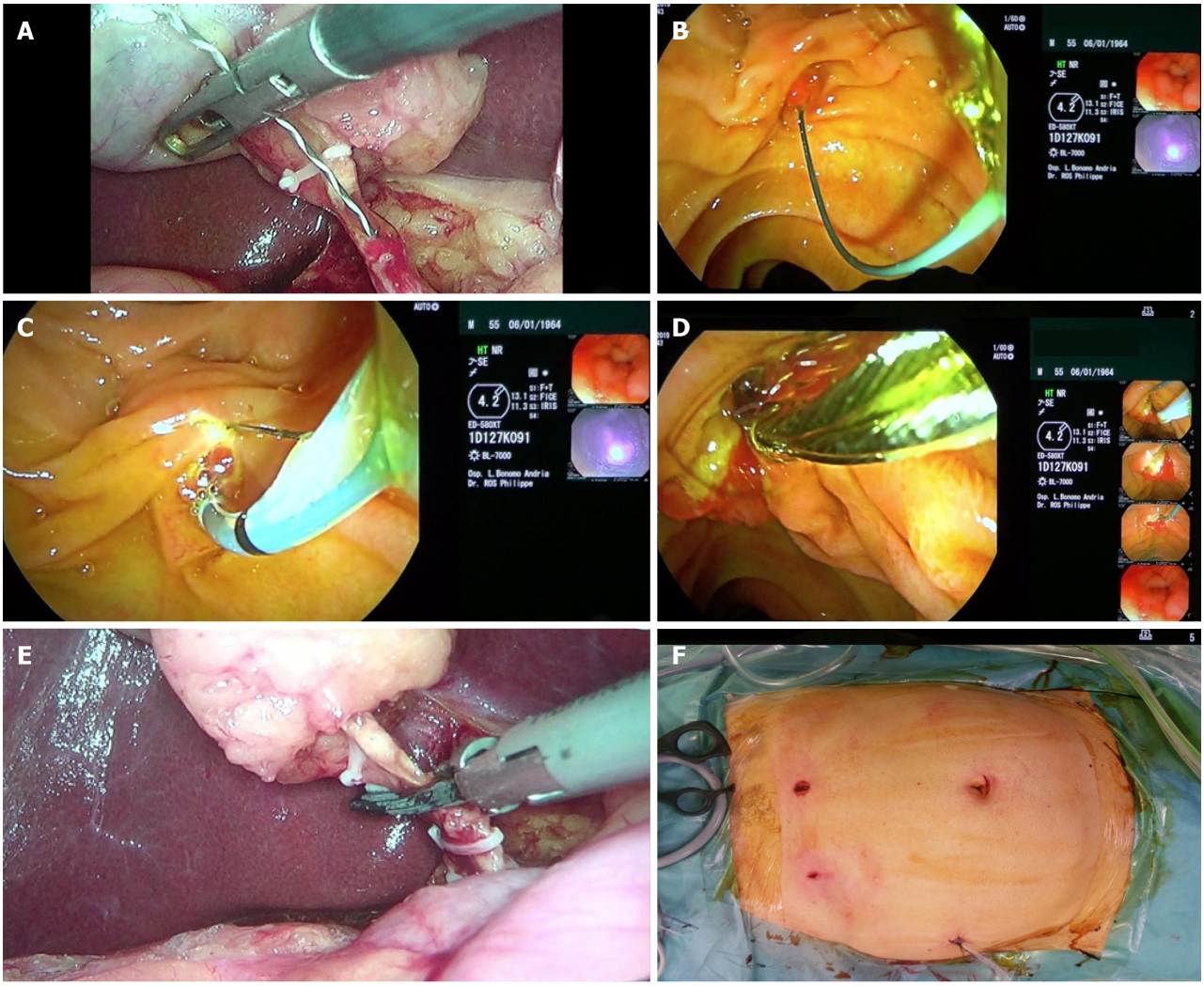


Figure 2 Intraoperative images: Endoscopic retrograde cholangio-pancreatography during laparoscopic cholecystectomy ("rendezvous technique"). A: Insertion of the guide wire into the cystic duct; B: Guide wire exits through the papilla into the duodenum; C: Endoscopic sphincterotomy on guide wire; D: Extraction of stones from the common bile duct with a dormia basket; E: Section between clips of the cystic duct and subsequent retrograde cholecystectomy; F: Postoperative final scars.

Extracorporeal shock-wave lithotripsy is also used in cases of difficult CBDS with US or fluoroscopic guidance. Modern lithotriptors employ water-filled compressible bags; because of the discomfort experienced by the patient, general anaesthesia is often required. Contraindications to this technique are portal thrombosis and varices of the umbilical plexus, and it can also cause adverse events, such as transient biliary colic, subcutaneous ecchymosis, cardiac arrhythmia, self-limited haemobilia, cholangitis, ileus, and pancreatitis[152,153]. More sessions are typically required. The recurrence rate during a 1-2 year follow-up period is about 14%[154]. Currently, extracorporeal shock-wave lithotripsy is not considered the first-line treatment for difficult bile duct stones[155].

CONCLUSION

Management of cholelithiasis with choledocholithiasis must be conducted appropriately. A delay in the diagnosis of this pathological condition can increase morbidity and mortality. Unlike other diseases that have a certain diagnosis, the presence of stones in the CBD is sometimes only suspicious. Historically, diagnosis was achieved through a careful association between clinical symptoms, serology, and radiological images. Today, the development of new radiological imaging, interventional endoscopy, and laparoscopy techniques has allowed us to arrive at a faster and more accurate diagnosis. The management of cholelithiasis with choledocholithiasis has become multidisciplinary, and more professional figures are involved

(radiologists, gastroenterologists, endoscopists, and surgeons), and will be increasingly adapted not only to a specific patient but also to the available resources of a specific environment in order to have the best possible management. However, endoscopy and surgery always retain a central diagnostic and therapeutic role.

Many studies and meta-analyses have been conducted by various authors regarding the comparison between one-session and two-session treatments for patients with concomitant gallbladder and CBD stones[17,31,76,92,156,157]. The findings have shown equivalent success rates, postoperative morbidity, stone clearance, mortality, conversion to other procedures, total operation time, and failure rate, but one-session treatment is characterized by a shorter hospital stay and more cost benefits[158]. Consequently, the latter option, when local resources and expertise are available, should be offered as a treatment of choice. However, in cases of incomplete or difficult removal of CBDSs, other additional techniques can also be used, which can be of valuable help in selected patients.

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REVIEW

Cellular factors involved in the hepatitis C virus life cycle

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Abstract

The hepatitis C virus (HCV), an obligatory intracellular pathogen, highly depends on its host cells to propagate successfully. The HCV life cycle can be simply divided into several stages including viral entry, protein translation, RNA replication, viral assembly and release. Hundreds of cellular factors involved in the HCV life cycle have been identified over more than thirty years of research. Characterization of these cellular factors has provided extensive insight into HCV replication strategies. Some of these cellular factors are targets for anti-HCV therapies. In this review, we summarize the well-characterized and recently identified cellular factors functioning at each stage of the HCV life cycle.

Key Words: Hepatitis C virus; Cellular factor; Viral entry; Translation; Replication; Assembly; Release

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Core Tip: The hepatitis C virus (HCV) depends on its host cells to propagate successfully. Hundreds of cellular factors involved in the HCV life cycle have been identified. Some of these cellular factors are potential targets for anti-HCV therapies (*e.g.*, scavenger receptor class B type 1, epidermal growth factor receptor, Niemann–Pick C1-like 1, microRNA-122, cyclophilin A). A successful vaccine for HCV is still a challenge in the near future. Investigating the cellular factors involved in viral entry should help vaccine development. HCV is also a unique model to study the interactions between viral infection and cellular lipid metabolisms.

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INTRODUCTION

Around 50%–80% of patients infected with the hepatitis C virus (HCV) will develop chronic infection. Approximately 71 million individuals are chronically infected by the HCV worldwide according to an estimation by the World Health Organization (https://www.who.int/hepatitis/publications/global-hepatitis-report2017/en/).

Chronic hepatitis C (CHC) patients are at high risk of developing liver cirrhosis and even hepatocellular carcinoma. Although CHC can now be cured using various direct-acting antivirals, the majority of CHC patients remain undiagnosed and, thus, untreated. Furthermore, a successful antiviral treatment does not prevent HCV reinfection. Therefore, HCV eradication remains a challenge and will probably require an effective vaccine[1] (for a review see References[2,3]).

HCV belongs to the family *Flaviviridae* and genus *Hepacivirus*. Its genome is a single-stranded RNA with positive polarity that is packaged by viral core protein and enveloped by a lipid membrane containing two viral glycoproteins (*i.e.*, E1 and E2) to form the virion. HCV genomic RNA sequences are highly heterogeneous among different isolates. At present, HCV is classified into at least six major genotypes (1 to 6). HCV heterogeneity hinders the development of an effective vaccine to protect against infection from all HCV genotypes. Despite the sequence variations, all currently recognized HCV genotypes are pathogenic, hepatotropic and preserve the similarity of the life cycle in cells (for a review see References[4,5]).

The HCV life cycle begins with the binding of a virion to its specific entry factors (or receptors) on hepatocytes. After binding, the virion is internalized into the cytoplasm and its genomic RNA is released. Then, the HCV genomic RNA is used for both polyprotein translation and viral replication. HCV replication takes place within the membranous web (MW) in the endoplasmic reticulum (ER). At last, HCV uses the biosynthetic pathway of very-low-density lipoprotein (VLDL) to assemble and release the viral particles from cells (for a review see Reference[6]).

HCV relies significantly on its host cells to establish a successful infection due to the fact of its limited genetic content. Hundreds of cellular factors have been identified as being involved in the HCV life cycle over more than thirty years of research. In this review article, due to the limited space, we can only summarize the well-characterized and recently identified cellular factors involved in the HCV life cycle but not including immune responses against viral infections (for a review see Reference[7]).

HCV PARTICLES

It has been demonstrated that purified HCV particles are spherical and heterogeneous in size (40–100 nm in diameter) with no obvious symmetry under cryo-EM[8]. The density of HCV particles in the plasma of CHC patients also varied (from 1.03 to 1.25 g/mL). Low-density HCV particles with 81–85 nm diameters are highly infectious[8,9]. The HCV particles with low density are associated with apolipoproteins (Apo such as Apo-E, Apo-AI, Apo-CI and Apo-B), phospholipids (such as phosphatidylcholine and sphingomyelin) and cholesterol (free and esterified), while they have almost no phosphatidylserine (PS) and very little amounts of phosphatidylethanolamine[8,10-12]. HCV particles derived from cultured cells show characteristics similar to those from CHC patients[9,10,13]. Thus, these circulating HCV particles are referred to as "lipo-viro particles" (LVPs)[14] and have very low buoyant densities due to the fact of their interactions with lipoproteins in the blood[15] (Figure 1) (for a review see Reference[16]).

Modifications of LVPs could be mediated by lipoprotein lipases (LPLs)[17,18]. LPL was shown to shift the HCV particles to higher densities with diminished HCV infectivity[19,20]. Indeed, LPL lipolytic activity in clinical samples has been reported to be inversely correlated with HCV viremia[21].

More than 40 cellular proteins were found in the HCV particles recently using proteomics analyses, including heat shock cognate protein 70 (HSC70) and nucleoporin 98 (Nup98); both proteins play a role in virus assembly/release[22,23].

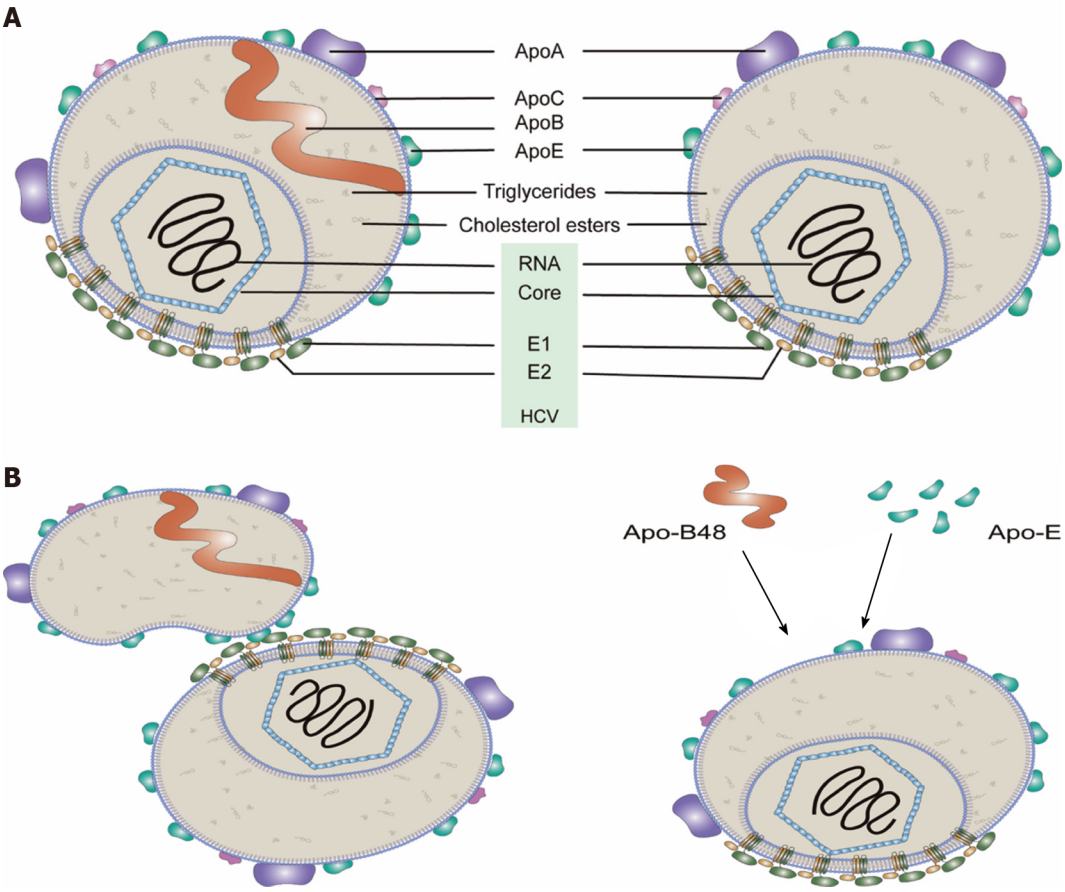


Figure 1 Model of hepatitis C virus particles (lipo-viral particles) secreted from cells. A: Most of the lipo-viral particles (LPV)'s membrane is a lipid monolayer. A bilayer-containing region is where the viral envelope proteins (*i.e.*, E1 and E2) are inserted. Viral envelope proteins may also exist in the phospholipid monolayer membrane. Though the precise structure has not yet been determined, LVPs are believed to have multiple copies of Apo-E and less Apo-A1 molecules but only one Apo-B100 molecule (left). Some LPVs may not have Apo-B (right). Within the phospholipid monolayer, there are the core proteins wrapping the viral RNA genome and neutral lipids (*e.g.*, cholesterol esters and triglycerides); B: Serum lipoproteins are possibly associated with hepatitis C virus particles in different ways. HCV: Hepatitis C virus.

Association of HCV particles with host lipoproteins has advantages for the virus: (1) Lipoproteins likely mask viral epitopes of viral E1 and E2 proteins thus helping the virus escape from the humoral immune response; and (2) Lipoproteins contribute to the hepatotropism of HCV, *e.g.*, HCV attachment to hepatocytes involving Apo-E (and/or Apo-B) binding with the cellular factors [*e.g.*, low-density lipoprotein receptor (LDLR), highly sulfated heparan sulfate proteoglycans (HSPGs) and scavenger receptor class B type 1 (SR-B1)] for viral entry[24].

VIRAL ENTRY

HCV can infect hepatocytes and other cells (*e.g.*, lymphocytes). In general, HCV infects hepatocytes through two distinct routes: Cell-free virus entry and cell-to-cell transmission. Viral entry requires interactions between the components of LPVs (particularly Apo-E and viral envelope proteins) and those of cells (particularly entry factors). Alternatively, HCV may infect cells through exosomes (for a review see Reference[25]).

Cell-free virus entry into hepatocytes

HCV transmission is through the parenteral route, and it needs to reach the liver by crossing the endothelium. Before attachment to the hepatocytes, HCV particles may be captured by DC-SIGN on dendritic cells or L-SIGN on endothelium[26,27] (Figure 2).

Cellular factors responsible for HCV entry into hepatocytes are divided into three groups based on their functions[25,28]: Attachment factors, entry factors (or receptors) and entry cofactors (or facilitators). Attachment factors help dock the virus on the cell's surface, mostly through non-specific interactions. Entry factors mediate specific

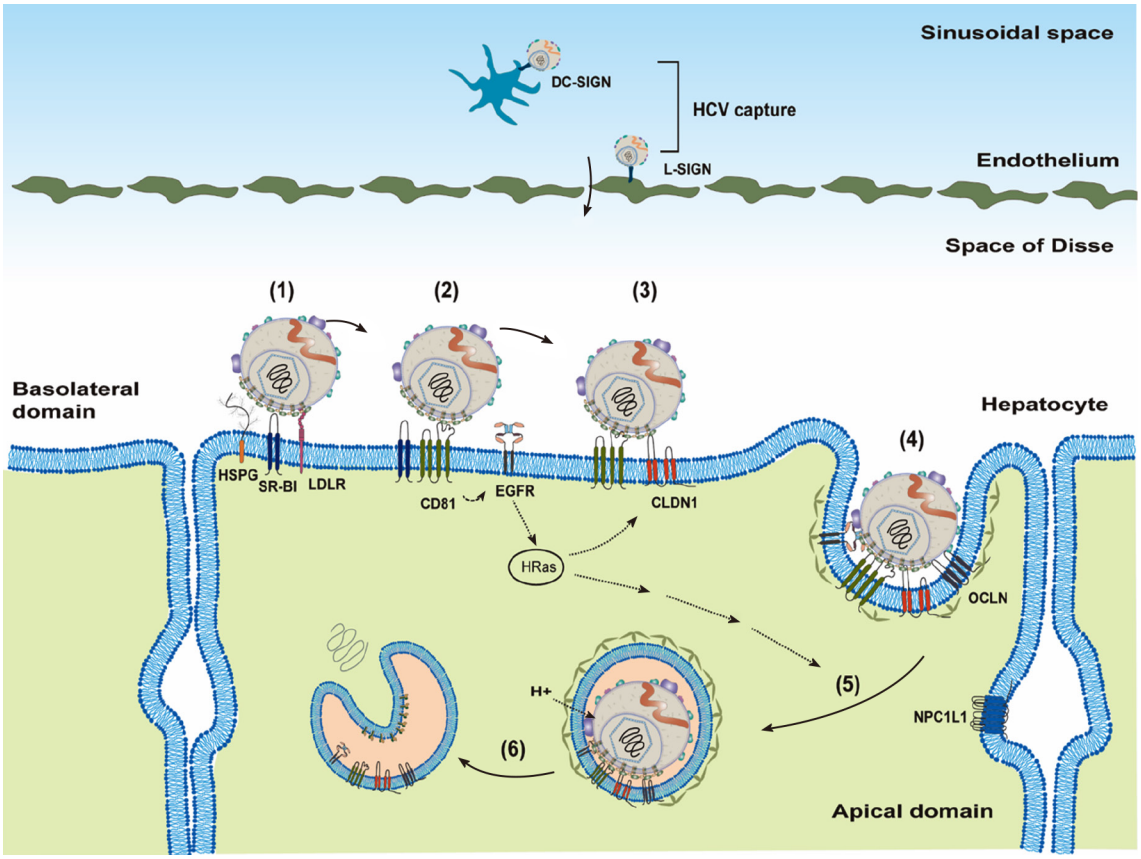


Figure 2 Model of cell-free virus entry into hepatocytes. Hepatitis C virus (HCV) lipo-viral particles (LPVs) may be captured by DC-SIGN on the dendritic cells or L-SIGN on the endothelium in the sinusoidal space. After transfer to Space of Disse, HCV LPVs could attach to the hepatocytes through interacting with highly sulfated heparan sulfate proteoglycans, low-density lipoprotein receptor and scavenger receptor class B type 1 (1). This attachment allows the engagement of LPVs to cluster of differentiation 81 (CD81) and then induces the epidermal growth factor receptor receptor signaling (2). Lateral diffusion of the CD81–HCV complexes results in the association of CD81–HCV with Claudin-1 (3) and then OCLIN (4). Formation of the HCV–CD81–CLDN1–OCLIN complex allows viral particles internalized through clathrin-dependent endocytosis (5). Endosomal acidification induces the fusion of viral particles possibly through E1 and leads to the release of the viral genomic RNA into cytosol (6). HSPGs: Heparan sulfate proteoglycans; HCV: Hepatitis C virus; EGFR: Epidermal growth factor receptor; LDLR: Low-density lipoprotein receptor.

interactions with viral glycoproteins. Entry cofactors, although not interacting directly with the virus, play an important role in supporting viral entry.

To infect a new hepatocyte, the HCV needs to interact with the attachment factors on the basolateral side of hepatocytes first (Figure 2). The well-known attachment factors include HSPGs, (particularly syndecans: SDC-1 and SDC-2), LDLR and SR-B1 [29-32]. Attachment of HCV LPVs to host cells is mediated mainly through the virus-associated lipoprotein components (particularly Apo-E[33]) and viral glycoproteins (E1 and E2, particularly E2)[32,34,35]. In addition to the binding of Apo-E with attachment factors, Apo-B100 could also interact with LDLR[36] and Apo-AI with SR-B1[37]. Recently, the redundant functions of Apo-C1 and Apo-E in the HCV infection have been demonstrated[38]. Attachment to SR-B1 may bring HCV particles to entry factor cluster of differentiation 81 (CD81)[39]. SR-B1, as both an entry factor and an attachment factor, has been shown to bind viral envelope proteins[29,40]. Interaction of HCV with CD81 activates epidermal growth factor receptor (EGFR) signaling and also facilitates CD81 diffusion and formation of the HCV–CD81–CLDN1 complex[39, 41-43]. The HCV–CD81–CLDN1 complex then interacts with OCLN, which is believed to mediate the clathrin-dependent internalization through interacting with GTPase dynamin[44-46]. SR-B1, CD81, CLDN1 and OCLN are four well-characterized entry factors for HCV entry[28]. In addition, LRL-R could interact with both HCV and E2 proteins and, thus, function as an entry factor[47].

HCV particles are then internalized, mature in the acidic endosomes that promote low pH-dependent HCV fusion and, ultimately, release HCV genomic RNAs (uncoating) into cytosol[48-50] (Figure 2). Low endosomal pH and interactions of viral glycoproteins with CD81 are thought to induce conformational rearrangements of viral glycoproteins for HCV fusion[51], which is controlled by E1 protein[52]. Recently, cell death-inducing DFFA-like effector B (CIDEB) protein was identified as an entry

cofactor to act at a late membrane fusion event[53].

Both HCV and coronavirus [*e.g.*, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)][54] are positive-strand RNA viruses. These two viruses enter their target cells through receptor-mediated endocytosis and release their genomic RNAs for translation in the cytosol. However, unlike HCV, coronavirus fusion for viral entry is unusual in that it is often biphasic and can occur at or near the cell's surface or in late endosomes[55].

In addition to HSPGs, LDLR, and SR-B1, interferon receptor IFNAR2 was found as a novel attachment factor to facilitate HCV entry through interacting with Apo-E[56]. VLDL receptor (VLDLR), similar to LDLR, could also serve as an attachment factor for HCV entry[57]. Niemann–Pick C1-like 1 (NPC1L1) contributes to HCV entry possibly through its role as a cholesterol receptor, thus functioning as an HCV entry cofactor [58]. Similarly, PS receptor (TIM-1/human hepatitis A virus cellular receptor 1/CD365) has also been identified as an attachment factor through binding with PS on HCV LPVs[59,60]. It should be noticed that only tiny amounts of PS were detected in the HCV particles[12]. On the other hand, LPLs, hepatic triglyceride lipase and long-chain fatty acyl-coenzyme A can inhibit HCV attachment by targeting virus-associated lipoproteins[17,18,20,61].

In addition to CLDN1, other CLDNs (*e.g.*, CLDN6, CLDN9 and CLDN12) have also been reported as HCV entry factors for some genotypes[62-64]. Recently, CD63 (binding directly with HCV) and CD36 (interacting directly with HCV E1 protein) were demonstrated to be entry factors[65,66]. Under hypoxic conditions, VLDLR could serve as an entry factor independent of canonical CD81-mediated HCV entry[67].

Most cellular factors, not directly interacting with the virus, involved in viral entry are served as the function of entry cofactors[25]. Some entry cofactors were identified to interact with SR-B1 [*e.g.*, PDZK1 and UDP-glucose: Glycoprotein glucosyltransferase 1 (UGGT1)]. The scaffold protein PDZK1 may be involved in linking SR-B1 to the actin cytoskeleton and the endocytic network[68], while UGGT1, a key component of the calnexin cycle involved in protein glycosylation, may stabilize SR-B1[69].

In addition to EGFR signaling, the association of CD81–CLDN1 is also regulated by protein kinase A (PKA)[70], ephrin receptor A2[71] and phosphatidylinositol 4-kinase type III-alpha/beta[72]. These are also entry cofactors supporting viral entry. Several other entry cofactors were identified as CD81-associated factors including HRas[39], integrin 1 (ITGB1)[39], Ras-related protein Rap2B[39], calpain-5[73], ubiquitin ligase Casitas B-lineage lymphoma proto-oncogene B[73] and serum response factor binding protein 1[74]. The membrane lipid composition has been shown to affect CD81 expression on the cell's surface and, in turn, modulate HCV entry[75]. Indeed, depleting cholesterol from the plasma membrane has been shown to decrease viral entry owing to reduced CD81 on the cell's surface[76]. In agreement with these findings, lipid-free Apo-E was demonstrated to induce adenosine triphosphate-binding cassette subfamily G member 1 protein-dependent cholesterol efflux and inhibit HCV replication[77].

Several other entry cofactors affecting CLDN1 and/or OCLN localization are also important in the HCV entry. E-cadherin, tumor-associated calcium signal transducer 2 (TACSTD2) and possibly Rab13 could regulate the localization of CLDN1 and OCLN [78-80]. Cell surface localization of CLDN1 is also regulated by vesicular transport proteins (such as Sec24C)[81]. Serotonin 2A receptor (5-HT2AR) is shown to control CLDN1 localization through PKA-mediated phosphorylation[82].

Several cellular factors involved in the process of HCV internalization have been identified: the expression level of SR-B1[83,84], EGFR–MKNK1 signaling[85,86], Abl tyrosine kinase[87], signaling pathways of Rac/Rho/CDC42/mitogen-activated protein kinase[41], actin[50], transferrin receptor 1[88], adaptor proteins (AP)-2-associated protein kinase 1[89] and cyclin G-associated kinase[89].

Pim kinase[90], Ankyrin repeat domain 1[91] and solute carrier family 3 member 2 [92] proteins are required for HCV entry, though their exact roles are not yet defined. In addition to the clathrin-dependent internalization pathway, HCV could also use clathrin-independent mechanisms to enter different cells[93].

Viral cell-to-cell transmission

In addition to cell-free virus entry into hepatocytes, HCV particles can also transmit directly from an infected hepatocyte to an adjacent hepatocyte[94-96]. A recent study demonstrated that the HCV core, *E1*, *E2* and *P7* genes were essential for not only cell-free viral transmission but also cell-to-cell transmission[97]. Several cellular factors were reported to contribute to HCV cell-to-cell transmission, including CD81[98,99], SR-B1[95], CLDN1[98], OCLN[98], LDLR[98], SDC-1[98], SDC-2[98], TIM-1[98], Apo-E in mature HCV particles[33,98,100], AP-1B and AP-4[101]. However, SR-B1-

independent[102] or CD81-independent[103] HCV cell-to-cell transmission has also been reported. More studies are needed to clarify this issue.

Viral transmission through exosomes

Exosomes, small vesicles normally used for intercellular communication, have been reported containing HCV RNA[104-107]. HCV transmission through exosomes should be independent of any entry factor, and it is resistant to antibody neutralization. However, further investigation is required to understand the mechanisms of transmitting HCV genomic RNA through exosomes[108].

Infection of HCV into other cells

B7.2 (CD86)[109] enabling lymph-tropic HCV to infect memory B cells and CD5[110] important for HCV entry into human T lymphocytes were identified. HCV enters macrophages mainly through phagocytosis[111].

VIRAL PROTEIN TRANSLATION

After the fusion between the viral envelope and the endosomal membrane, HCV positive-strand RNA will be released into the cytosol (Figure 2). Translation of HCV genomic RNA is modulated by the viral RNA structure, cellular translational machinery [*e.g.*, 40S, translation initiation factors (eIFs)] and several cellular regulatory factors [*e.g.*, microRNA-122 (miR122) and internal ribosome entry site (IRES) trans-acting factors (ITAFs)] (for a review see Reference[112]).

The most important structures of the HCV genomic RNA for translational regulation reside in the IRES of the 5'-untranslated region (5'UTR), a short segment of the core gene sequence, the cis-acting replication element (CRE) in the NS5B coding region[113] and the entire 3'UTR[114] (Figure 3). Many long-range RNA–RNA interactions among different regions of the HCV genomic RNA are involved in translational regulation[115]. The HCV IRES could bind to the ribosomal 40S subunit directly[116-118]. Interaction between the viral IRES and cellular 18S rRNA consisting of a three-nucleotide base pairing of these two molecules is crucial for HCV translation [119].

When eIF2–Met–tRNAi Met is available, canonical eIFs, including eIF3, eIF2, eIF1A, eIF5 and eIF5B, can initiate HCV translation[120]. On the other hand, when eIF2–Met–tRNAi Met is limited, eIF2A[121], eIF2D[122], eIF5B[123], a combination of eIF2A and eIF5B[124], or MCT-1 and a DENR protein complex[125] could substitute for eIF2 to initiate the translation (for a review see Reference[112]). A single RNA loop in domain II of the HCV IRES controls the translation from initiation to elongation [126].

The most important cellular factor involved in HCV translation is miR122. The liver-specific miR122 is crucial for HCV production (for a review see Reference[127]). miR122 can bind to two adjacent recognition sites on the HCV 5' UTR with the help of human Argonaute protein 2 (hAgo2)[128-130]. The hAgo2/miR122 complex could alter the HCV 5'UTR structure and promote the IRES formation to enhance viral translation[131].

Several cellular proteins could bind to the HCV 5'UTR directly as ITAFs and enhance the viral protein translation such as La[132], heterogeneous nuclear RNA-interacting protein Q (hnRNP Q; NSAP1)[133], hnRNP L[134], hnRNP D[135], IGF2BP1 (insulin-like growth factor 2 mRNA binding protein 1; IMP-1)[136], poly(C)-binding protein 2 (PCBP2)[137], the LSm1-7 complex[138] and polypyrimidine tract binding protein (PTB)[139]. On the other hand, RNA binding protein 24[140] and Gemin5 would possibly suppress HCV translation[141].

Some proteins do not bind to HCV RNA directly but act through protein–protein interactions to help HCV translation, such as HuR (ELAVL1)[142], and the proteasome subunits[143].

Several other cellular proteins, identified as positive regulators of HCV translation through different mechanisms include MAP kinase interacting serine/threonine kinase 1 (MKNK1), phosphatidylinositol 4-kinase catalytical subunit beta (PI4K-beta) [144], ubiquitin-specific protease 15[145], NSAP1[146], RNA helicase DDX6 (RCK, p54) [147] and heat shock protein 70[148].

In addition to miRNAs and proteins, long non-coding RNA (lncRNA), such as HULC, could enhance HCV translation[149]. Moreover, the PI3K–Akt signal pathway could also upregulate HCV translation through the activation of SREBPs[150].

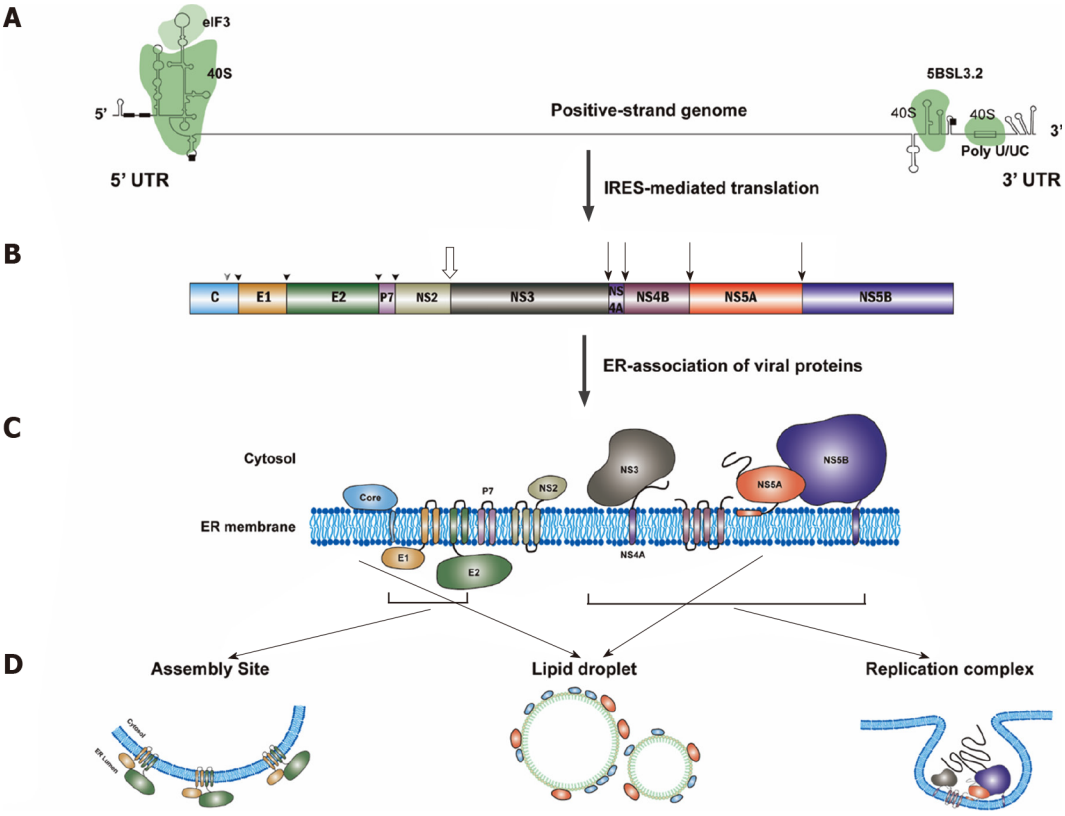


Figure 3 Hepatitis C virus protein translation. A: Translation of hepatitis C virus (HCV) genomic RNA is regulated by the internal ribosome entry site in the 5'-untranslated region (5'UTR) along with a short segment of the core gene sequence, the cis-acting replication element in the NS5B coding region and by the entire 3'UTR. Binding sites for eIF3 and 40S were marked. The start and stop codons for protein translation are marked by black squares, while two recognition sites on the 5'UTR for miR122 are marked by black rectangles; B: Polyprotein is co- and post-translationally cleaved by host or viral proteases to yield the structural proteins (core, E1 and E2) and the nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B proteins). Core, E1 and E2 are processed by cellular signal peptidase (filled arrowhead). Mature core protein will be generated after further cleavage by signal peptide peptidase (empty arrowhead)[317]. The NS2/NS3 junction site is cleaved by the NS2–NS3 auto-protease[318] (empty arrow), and the remaining nonstructural proteins are processed by the NS3/4A proteinase[319] (filled arrow); C: All of the HCV proteins are associated with endoplasmic reticulum directly or indirectly[320]; D: Then, NS3, NS4A, NS4B, NS5A and NS5B proteins will form the replication complex. Core and NS5A proteins will be transferred to lipid droplets, while E1 and E2 proteins will stay in the assembly sites. IRES: Internal ribosome entry site; 5'UTR: 5'-untranslated region.

Expression of the alternative reading frame/core+1 protein

In addition to the synthesis of HCV polyprotein, another viral protein is produced through the core+1 reading frame (for a review see References[151,152]). Recently, the conserved RNA stem loops (SLs) SL47 and SL87 of the HCV core gene were identified to possess a novel cis-acting element and direct the internal translation initiation of the alternative reading frame (ARF)/core+1[153].

RNA REPLICATION

HCV genomic RNA replication, tightly linked to ER membrane alterations designated the MW[154,155], occurs in the replication organelles (ROs)[156], mainly consisting of double membrane vesicles (DMVs)[157]. The HCV RNA replication requires at least viral genomic RNA and viral RNA-dependent RNA polymerase (NS5B protein) in the ROs. Thus, HCV RNA replication could be modulated by the viral RNA structure (*i.e.*, CREs), viral proteins (particularly, NS5B) and the biogenesis of ROs. Many cellular factors play important roles in the modulation of HCV RNA replication (for a review see References[158,159]).

The CREs of HCV genomic RNA were identified close to the 5'UTR and 3'UTR of the genome[160]. Several cellular proteins could bind to the 5'UTR and the 3'UTR of the viral RNA, facilitate the genome circularization and enhance RNA replication. These proteins include La[161], hnRNP L[162], the NFAR protein complex (NF90, NF45, and RHA)[163], PTB[164], PCBP2[137], and RNA binding protein 24[140]. High-mobility group box 1 interacting with SL 4 of 5'-UTR[165], Src-associated in mitosis 68 kDa protein binding with SL2 of 5'-UTR[166] and HSC70 with poly U/UC in the 3'-

UTR[167] could also promote HCV replication.

In addition to cellular proteins, liver-specific miR122 could bind to the two adjacent sites of HCV 5'UTR[168], forming a ternary complex[169]. Through this interaction, in addition to stimulating translation[128], miR122 could protect the genome from cellular DUSP11 pyrophosphatase activity[170] and subsequent degradation by the exonucleases Xrn1[171,172] and Xrn2[172,173] to facilitate RNA replication. Cellular proteins involved in HCV RNA replication through the regulation of miR122 include DDB1-Cul4 associate factor 1[174], glycogen synthase kinase 3b[175], GTPase Rab27a [176], heterogeneous ribonucleoprotein K (hnRNP K1)[177] and DEAD-box RNA helicase (DDX6)[178]. Interestingly, various miRNAs were found to compensate for the role of miR122 on HCV replication in non-hepatic cells[179].

The HCV nonstructural proteins, NS3 to NS5B, are required for RNA replication, and NS5B constitutes the catalytic core of the HCV replication complex[180,181]. Cellular protein kinase C-related kinase 2 could phosphorylate NS5B to regulate HCV RNA replication[182]. Several cellular factors were identified to enhance HCV RNA replication through interacting with NS5B, including cellular chaperonin TRiC/CCT [183], ribonucleotide reductase M2[184], sphingomyelin[185], HuR[186], VAPB-MSP [187] and CYP4F12[188].

In addition to NS5B, NS5A is also essential to HCV RNA replication. DDX3[189], Y-box binding protein 1[189] and FKBP6[190] could interact with NS5A to facilitate HCV RNA replication. Cellular cyclophilin A (CypA)[191,192] and human replication protein A[193] could bind to NS5A and stimulate the binding of NS5A to NS5B and viral RNA to facilitate HCV RNA replication. Three domains in NS5A protein were identified using biochemical analyses: Domain I (a.a. 1–213), domain II (a.a. 250–342) and domain III (a.a. 356–448) in NS5A[194]. Domain I, with RNA-binding ability, is essential for RNA replication[195], while domain III is required for assembly[196]. A critical ratio between the different phospho-forms of NS5A protein must be maintained for productive HCV RNA replication[197]. Several cellular kinases could phosphorylate HCV NS5A, including casein kinase II (CKII)[198] and CK1a[199], lipid kinase PI4KIIIα[200] and c-Abl tyrosine kinase[201]. These kinases control the switch between virus replication and assembly by phosphorylating NS5A[202]. Vinexin b also regulates HCV replication *via* modulating the phosphorylation status of NS5A in a CK1a-dependent manner[203]. Notably, association of vesicle-associated membrane protein A (VAP-A) with NS5A depends on the phosphorylation status of NS5A[204]: VAP-A binds to hypophosphorylated NS5A and contributes to HCV RNA replication [202]. F-box/LRR-repeat protein 2, when geranylgeranylated, could also interact with NS5A and promote HCV replication[205]. On the other hand, proprotein convertase subtilisin/kexin type 9 interacts with NS5A and inhibits HCV replication, possibly through preventing the dimerization of NS5A[206]. SPRY domain- and SOSC box-containing protein 2 induces NS5A ubiquitination and degradation to suppress HCV replication[207].

Viral NS3 protein is also an important component of HCV replication complex. Rad51[208] and GBF1[209] could interact with NS3 and promote HCV RNA replication.

HCV NS3–NS5B proteins, in collaboration with cellular factors, could induce MW formation. Among these viral proteins, NS5A and NS4B play a major role in the induction of MW[210,211]. Cellular factors could modulate HCV replication through affecting MW biogenesis. For example, CypA plays a role in the formation of DMVs through interacting with NS5A, in addition to its role in facilitating HCV RNA replication[212]. Similarly, receptor for activated protein C kinase 1 and ATG14L were found to participate in the DMV formation through interacting NS5A[213].

Proline–serine–threonine phosphatase interacting protein 2, a protein with membrane-deforming activity, is critical for MW formation through directly interacting with NS4B and NS5A[214]. Cytosolic phospholipase A2 gamma group IVC (PLA2G4C) is also required in the biogenesis of the MW. PLA2G4C expression was upregulated after HCV infection, contributing to HCV replication and assembly through interacting with NS5A and NS4B[215].

The cellular protein Surf4, maintaining ER-Golgi intermediate compartments and the Golgi compartment, was recruited into ROs by NS4B and was involved in the formation of DMVs[216]. Another cellular protein prolactin regulatory element binding could promote HCV RNA replication by interacting with NS4B and participating in the formation of DMVs[217].

HCV replication also depends on the GBF1-Arf1-COPI complex[218,219] and phosphatidic acid phosphatase lipin1[220], possibly due to their involvement in the MW biogenesis. Sphingomyelin and ceramide transfer protein (CERT), which is in the sphingomyelin biosynthesis pathway, are also essential for the biosynthesis of DMVs

[221].

Several Rabs (the Ras superfamily of small GTPases) are involved in the formation of HCV RNA replication machinery: (1) Rab5 can be recruited by NS4B and involved in HCV genome replication[222]; (2) Rab5 and 7 co-localize with NS4B and Rab2, 5 and 7 are required for HCV RNA replication[223]; (3) Rab1b and its negative regulator TBC1D20 are involved in the HCV replication[224,225]; and (4) Rab18, through associating with NS5A directly, is believed to promote the physical interaction between LDs and Ros[226].

Proteins in the nuclear transport machinery [including soluble nuclear transport factors, *e.g.*, karyopherins (Kaps)] and nucleoporins (Nups) in the nuclear pore complexes are involved in the HCV life cycle[227,228]. Interaction of various HCV proteins with Nups and Kaps could potentially alter host cell nucleocytoplasmic transport to facilitate HCV replication[229].

Several studies have demonstrated that autophagy plays an early role in establishing HCV re-plication[230,231]. Indeed, DMVs induced by HCV accumulate at the MW and morphologically resemble autophagosomes[232]. Thus, autophagy may help to induce the MW formation during HCV replication[233]. However, the role of autophagy in HCV replication is still a matter of controversy[234] (for a review see Reference[235]).

To shape an ER membrane into an RO requires not only viral and cellular proteins but also lipid synthesis (for a review see Refenence[236]). Many studies have shown that HCV could modulate lipid metabolism (*e.g.*, cholesterol and fatty acid biosynthesis) to promote viral replication[237-239]. Furthermore, to achieve robust HCV replication, it is necessary to limit the oxidative degradation of lipids[240]. Sphingolipid is also required for HCV replication and might contribute to detergent resistance of HCV replication sites[241]. Modulation of the lipid environment of RO by HCV includes the recruitment and activation of the lipid kinase PI4KIIIα by NS5A and NS5B proteins to generate enhanced levels of phosphatidylinositol 4-phosphate (PI4P) at the RO[200]. PI4P could attract lipid transport proteins [oxysterolbinding protein (OSBP), four-phosphate adaptor protein 2 and CERT)] to deliver glycosphingolipids, cholesterol and ceramide respectively to the RO[242,243]. OSBP and CERT could interact with the human VAP residing in the ER[244]. Both VAP-A and VAP-B, enriched in purified DMVs[245], interact with NS5A and NS5B and assist in the formation of the replicase complex[204,245]. Two types of lncRNAs, lin-IGF2-AS and lnc-7SK, are involved in HCV replication through regulating PI4P[246]. Recently, HCV NS3/4A protease was reported to control the activity of 24-dehydrocholesterol reductase, catalyzing the conversion of desmosterol to cholesterol, to regulate the lipid environment for HCV RNA replication[247].

FUSE binding protein 1 is reported to be an essential cellular factor required for HCV replication through inhibiting the function of tumor suppressor p53[248]. Several other cellular factors were involved in the HCV RNA replication, mTORC1[249] and chloride channel[250], but their exact roles are not yet defined.

VIRAL ASSEMBLY AND RELEASE

Assembly of HCV particles requires a viral genomic RNA, core proteins (for the capsid formation) and the viral envelope glycoproteins (E1 and E2). In addition to these viral factors, other viral nonstructural proteins and cellular factors, especially VLDL synthesis and secretion, are essential for the HCV assembly (for a review see Reference [251]).

Cleavage at the HCV core protein C-terminus by the intramembrane signal peptide peptidase is required for its maturation and targeting to LDs[252]. The mature core protein, forming the viral capsid, comprises two domains: The amino-terminal domain (D1; a.a. 1–118) and a central domain (D2; a.a. 119–177)[253]. D1 harbors basic aa residues that interact with viral RNA[254], while D2 is hydrophobic and associated with LDs[252]. LDs are important for the production of infectious HCV particles[255]. As expected, a reduction in the volume of LDs by the suppression of HSC70 expression[23] or disruption of LDs by the inhibitor of aryl hydrocarbon (AhR)[256] would inhibit HCV production. Thus, ADP-ribosylation factor-related protein 1 essential for LD growth is required for HCV propagation[257], while N-Myc Downstream-Regulated Gene 1 restricts HCV assembly by limiting LD formation[258].

Several cellular factors are involved in the association of the core with LDs. Diacylglycerol acyltransferase-1 (DGAT1) interacts with both the core and NS5A proteins and is required for the trafficking of these two proteins to LDs[259]. PLA2G4A also

plays a role in recruiting core to LDs, and its specific cleavage of lipids containing arachidonic acid is essential for the production of infectious viral particles[260]. Interaction of core and Nup98 in LDs is important for HCV propagation[22], while heterogeneous nuclear ribonucleoprotein K is recruited to sites in close proximity to LDs and suppress HCV production[261].

HCV genomic RNAs synthesized by the HCV replication complex (NS3–NS5B proteins) in the DMVs will be transferred by NS5A and NS3-4A proteins and encapsidated by the viral capsid to form the nucleocapsid. The HCV RNA structure [262] responsible for its encapsidation by core proteins has been suggested to be (1) a highly conserved secondary structure within the core D2 region[263]; (2) the conserved apical motifs of the 3'X region[264]; or (3) multiple RNA motifs with a secondary structure[265].

Lipid mobilization from cytoplasmic LDs favors the morphogenesis and secretion of HCV particles[266,267]. HCV infection suppresses the cellular lysophosphatidylcholine acyltransferase 1 expression resulting in altered lipid metabolism and, in turn, increases the production of infectious viral particles with low density[267]. α/β hydrolase domain-containing protein 5/CGI-58[266] and ATGL lipase[268] also mobilize lipids in LDs for the production of HCV particles.

PLA2G4C[215] and AAM-B[269] recruit the NS4B protein to LDs. Thus, these two proteins may bridge the steps of HCV RNA replication and assembly by translocation of RCs to LDs. In addition to DGAT1, CD2AP also participates in the transfer of NS5A to LDs[270]. Interactions between NS5A and core proteins are crucial for productive HCV infection[271]. Protein kinase C and CK substrate in neurons protein 2[272] and cortactin[273] promote interactions between HCV core and NS5A in the LDs. HCV NS5A protein domain I interacts with the D1 region of core protein[274]. Indeed, core and NS5A proteins are found associated with LDs at 12 h post-infection[275]. The LDs associated with core and NS5A proteins are close to the DMVs and the assembly sites on the ER membrane (Figure 4). Several studies suggested that the NS5A protein might link DMVs with assembly sites. Two LD-associated proteins, Rab18[226] and TIP47[276,277], were found to interact with NS5A and might help the juxtaposition of replication and assembly sites.

Formation of HCV nucleocapsid may occur in the LDs and/or assembly sites (Figure 4). Then, HCV nucleocapsid will move to the assembly sites and interact with viral E1/E2 proteins (envelopment) and bud into the ER lumen (egress) (Figure 4). All viral proteins are involved in HCV assembly[278,279]. The core and E1/E2 proteins are the integral protein components of an HCV particle. The other viral proteins do help viral assembly and egress, especially NS5A, p7, and NS2[278-281]. NS2, ubiquitinated by MARCH8[282], is a key regulator of viral assembly by bringing together the structural and nonstructural proteins required for particle formation. The cellular signal peptidase complex subunit 1 interacts with both NS2 and E2 proteins and mediates membrane association of the NS2–E2 complex to control HCV assembly [283]. Then, PLA1A plays a role in bridging NS2–E2 complex and NS5A-associated replication complex through its interaction with E2, NS2 and NS5A[284,285]. It is likely that NS2 protein brings E1, E2, NS3, NS5A and core proteins together to form a complex within the detergent-resistant membranes in the ER as an assembly platform to initiate HCV assembly[286]. Meanwhile, the clathrin Adaptor Related Protein Complex 2 Subunit Mu 1[287] and a small GTPase, Rab32[288], may transfer nucleocapsids to the sites of envelopment. HRS (hepatocyte growth factor-regulated tyrosine kinase substrate), an endosomal-sorting complex required for transport (ESCRT)-0 complex component, is involved in the viral envelopment[289].

HCV assembly and envelopment are linked to the VLDL synthesis and secretion [290]. Indeed, CIDEB, an important regulator of the VLDL pathway, contributes to the HCV assembly through interacting with NS5A[291]. However, inhibitors of microsomal triglyceride transfer protein (MTTP) affect secretion of HCV more severely than that of VLDL[292]. HCV is also reported to modify VLDL secretion[293,294]. These results suggest that HCV assembly occurs possibly through modification of the VLDL secretion. Indeed, colocalization of the core with Apo-E but not with Apo-B was demonstrated[295]. Therefore, it is more likely that HCV suppresses VLDL secretion and then uses the excess lipid to produce lipid-rich viral particles. Components of VLDL synthesis, such as MTTP[290], Apo-B[296] and especially Apo-E[10], have been implicated in HCV assembly. HCV production in HuH7 cells with double knockout of Apo-B/Apo-E was reduced significantly compared to that of single knockout cells, and ectopic expression of Apo-E in cells with double knockout of Apo-B/Apo-E restored production of infectious viruses. Furthermore, ectopic expression of Apo-E or MTTP in cells with double knockout of Apo-B/MTTP could restore infectious virus production[297]. These studies suggested that there are Apo-B-dependent and -

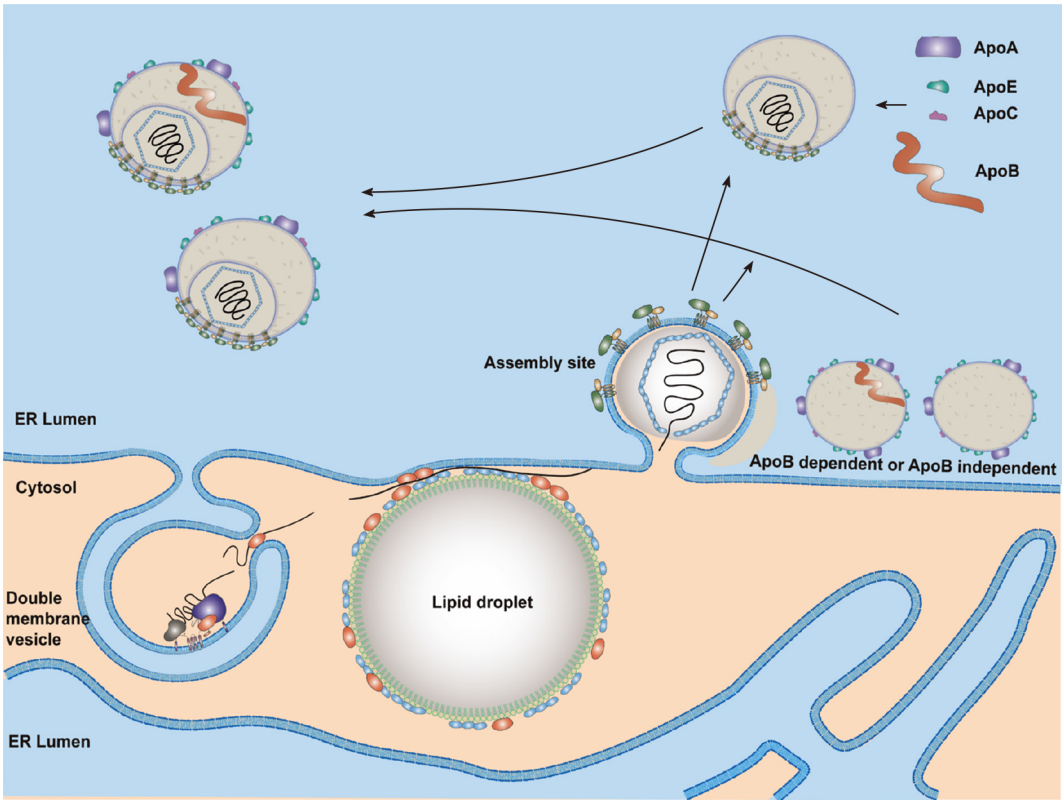


Figure 4 A proposed model for hepatitis C virus assembly. Lipid droplets (LDs) are surrounded by endoplasmic reticulum (ER)[321]. LDs with a hepatitis C virus (HCV) core and NS5A proteins are close to replication sites [double membrane vesicle (DMV)] and assembly sites. HCV genomic RNA synthesized by the replication complex (NS3–NS5B proteins) in the DMVs will be transferred by NS5A and NS3-4A proteins and encapsidated by the core proteins to form the nucleocapsid. Then, the HCV nucleocapsid will interact with glycoproteins E1/E2 in the assembly sites and bud into the ER lumen. Both Apo-B-dependent and -independent mechanisms are possibly involved in HCV particle assembly. One model shows the production of a fused form of HCV with very-low-density lipoproteins. Another model shows the budding of HCV particles with several apolipoproteins but not Apo-B. Nascent viral particles may be further lipidated by luminal lipoproteins and incorporated with exchangeable apolipoproteins. ER: Endoplasmic reticulum.

independent virus assembly pathways (Figure 4). Similar to the effect of ectopic expression of Apo-E in Apo-B/Apo-E double knockout cells, expression of exchangeable apolipoproteins (*e.g.*, Apo-A1, A2, C1, C2 and C3), the peptides of amphipathic α-helices containing the amino-terminal domain of Apo-E[297] or even human cathelicidin antimicrobial peptide[298] also restored infectious virus production. These results suggest that infectious virus production is regulated redundantly by exchangeable apolipoproteins expressed in the liver. Annexin A3 (ANXA3) through facilitating the incorporation of Apo-E[299] and Golgi protein 73, a resident Golgi membrane protein, through facilitating the interaction of HCV NS5A with Apo-E[300], promote HCV virion maturation. Recently, Apo-M, interacting with E2, was reported to be a novel virus particle-associated protein[301].

After envelopment, HCV particles then traffic to Golgi likely within COPII secretory vesicles[295,302]. Secretion of infectious HCV particles relies in part on components of the ESCRT pathway[303]. HCV egress but not VLDL secretion is blocked by silencing Rabs and the transGolgi network (TGN)-associating adaptors[304]. Moreover, inhibition of Apo-E secretion using monensin does not impair HCV release. These results suggested that HCV and VLDL use distinct secretion pathways[305]. Altogether, these results suggest that the release of HCV particles occur *via* a TGN-endosomal secretion pathway that is different from that of VLDL. The lipid-associated TM6SF2 (transmembrane 6 superfamily 2) has been demonstrated to promote lipidation and secretion of HCV particles[306]. The secreted HCV particle is likely a single particle fusion of viral structural proteins with various apolipoproteins and lipids (Figure 1).

Autophagy triggered by HCV-induced oxidative stress favors the release of HCV particles[307]. Thus, autophagy may play a role not only in the induction of DMVs but also in the secretion of HCV particles (for a review see Reference[235]).

HCV particles from the sera of HCV-infected patients harbor higher amounts of Apo-E than those derived from cell culture[308]. The interaction of Apo-E and HCV enhances specific infectivity and may aid HCV in evading neutralizing antibodies

[309]. HCV particles from the blood of HCV-infected patients contain Apo-B100 or Apo-B48, indicating that a significant fraction of HCV particles in blood is also associated with Apo-B48-containing lipoproteins[310]. These results suggested that the interactions between HCV particles and lipoproteins (*e.g.*, Apo-E and Apo-B48) in the blood of HCV patients (Figure 1B). Besides lipoproteins, specific serum factors, including albumin, also promote extracellular maturation of HCV particles[311].

Several cellular factors were involved in the assembly and secretion of HCV particles, such as ANXA2[312], sorcin (soluble resistance-related calcium-binding protein)[313], AP-1A, AP-1B, AP-4[101] and O-linked N-acetylglucosamine transferase [314], but their exact roles are not yet defined.

CONCLUSION

Study on the life cycle of the HCV has progressed tremendously after the development of *in vitro* HCV culture systems[315]. Understanding the HCV replication cycle led to the huge success of direct-acting anti-virals (DAAs) targeting NS3, NS5A, and NS5B. Hundreds of cellular factors involved in various stages of the HCV's life cycle have also been identified after more than 30 years of research on HCV–host cell interactions. Some of these cellular factors have been selected as targets for anti-HCV therapy (*e.g.*, SR-B1, EGFR, NPC1L1, miR122, CypA)[7]. Inhibitors against these cellular factors may complement existing DAAs. A successful vaccine for HCV is still a challenge in the near future. Understanding the mechanisms of viral entry, especially E2–CD81 interactions, should help in the development of a vaccine.

HCV particle, a hybrid lipo-viro-particle, does not look like a canonical enveloped virus. Thus, HCV has become a unique model for studying virus–host interactions, *e.g.*, between HCV and cellular lipid metabolisms. Furthermore, all positive-strand RNA viruses, including coronaviruses and picornaviruses, induce the reorganization of cellular membranes to replicate their genomes, similar to HCV[316]. Using HCV as a paradigm to study how HCV induces cellular membrane re-organization may lead to identification of broad-spectrum antivirals targeting cellular factors commonly used by these viruses.

Despite the impressive advances, many issues are still far from being clarified regarding HCV–host cell interactions. More studies are needed to understand the detailed mechanisms.

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REVIEW

Modulation of cell physiology under hypoxia in pancreatic cancer

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Abstract

In solid tumors, the development of vasculature is, to some extent, slower than the proliferation of the different types of cells that form the tissue, both cancer and stroma cells. As a consequence, the oxygen availability is compromised and the tissue evolves toward a condition of hypoxia. The presence of hypoxia is variable depending on where the cells are localized, being less extreme at the periphery of the tumor and more severe in areas located deep within the tumor mass. Surprisingly, the cells do not die. Intracellular pathways that are critical for cell fate such as endoplasmic reticulum stress, apoptosis, autophagy, and others are all involved in cellular responses to the low oxygen availability and are orchestrated by hypoxia-inducible factor. Oxidative stress and inflammation are critical conditions that develop under hypoxia. Together with changes in cellular bioenergetics, all contribute to cell survival. Moreover, cell-to-cell interaction is established within the tumor such that cancer cells and the microenvironment maintain a bidirectional communication. Additionally, the release of extracellular vesicles, or exosomes, represents short and long loops that can convey important information regarding invasion and metastasis. As a result, the tumor grows and its malignancy increases. Currently, one of the most lethal tumors is pancreatic cancer. This paper reviews the most recent advances in the knowledge of how cells grow in a pancreatic tumor by adapting to hypoxia. Unmasking the physiological processes that help the tumor increase its size and their regulation will be of major relevance for the treatment of this deadly tumor.

Key Words: Hypoxia; Cancer; Tumor; Pancreas; Proliferation; Cell survival

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Core Tip: Pancreatic ductal adenocarcinoma is characterized by high aggressiveness, therapeutic resistance, and mortality. The cells included in the mass, both tumor and those forming the stroma, have a high proliferative rate that leads to the rapid growth of the tumor. Because of this, the distribution of blood vessels is insufficient to supply oxygen to the cells, and hypoxia is a consequence. Cells escape from death and adapt by undergoing critical changes in intracellular pathways involved in energy supply, proliferation, and cell-to-cell communication.

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INTRODUCTION

World Health Organization estimates that about 465.000 people died from pancreatic cancer (PC) in 2020, which was the seventh leading cause of cancer deaths[1]. Various studies point toward recent increases in the incidence of PC and in the number of PC-related deaths[2,3]. The causes of high mortality are late diagnosis and few and ineffective therapeutic strategies. Of the various types of PC, pancreatic ductal adenocarcinoma (PDAC) accounts for 90% of the diagnoses[4].

Tumors conditions include the development of hypoxic areas of varying extent, in which the growing cell population is subjected to low O_2 availability[5]. This condition derives from the high rate of cell proliferation and the rapid growth of the tumor mass, which is accompanied by an abnormal distribution of blood vessels that limits blood flow and, hence the O_2 supply[6]. An ongoing hypoxic state has been well documented in PC. The average of O_2 level in the healthy pancreas is 6.8%, whereas in PC it is 0.4%. This 17-fold decrease in O_2 availability is high compared with other tumors[7]. Extensive fibrosis and hypovascularization are characteristic of PDAC, and lead to significant tissue hypoxia and the aggressiveness, therapeutic resistance, and high mortality that are characteristic of this type of cancer[8].

Not less important, the stroma that forms part of the tumor mass contributes to the creation of a tumor microenvironment that contributes to conditions that determine tumor growth, invasion, and metastasis[9]. The tumor microenvironment is composed of structural elements, which include extracellular matrix proteins and cells, such as macrophages, endothelial cells, fibroblasts, and stellate cells[9,10]. Pancreatic stellate cells (PSC) interact closely with cancer cells and set up a close relationship that favors tumor growth[11]. Additionally, PSC are considered major contributors to the fibrosis that forms part the stroma[12]. Increased hypoxia promotes collagen deposition and tumor progression, *i.e.* hypoxia contributes to the development of fibrosis in PC and in other types of tumors[13]. Hypoxia promotes PSC activation, increased proliferation, and invasiveness, confirming their participation in the formation of fibrotic tissue[14].

Hypoxia-inducible factor (HIF) plays a pivotal role in the development of cellular responses to hypoxia[15]. Usually, this factor promotes or represses the transcription of many of genes that are involved in cellular homeostasis. HIF is degraded, and hence is nonfunctional when O_2 is available to the cells, but becomes active under specific conditions, including low-O_2 (hypoxic) stress. The genes targeted by HIF allow cells to adapt to hypoxic conditions and survive. The genes involved code for vascular endothelial growth factor (VEGF), erythropoietin, and glucose transporter-1[16]. In order to adapt to O_2 deprivation, energy metabolism switches to glycolysis[17]. Concomitantly, regulation of the transcription of certain metabolic enzymes, including pyruvate dehydrogenase kinase 1, lactate dehydrogenase A, glycogen phosphorylase L, and others occur. The result is the improvement of adenosine triphosphate (ATP) production at the expense of increases of glucose uptake, glycolysis, and generation of lactate[18].

Several isoforms of HIF have been identified. HIF-1α and HIF-2α are closely related, and HIF-1α is the first isoform that was described. Both activate the transcription of genes associated with hypoxia. HIF-3α is more distantly related. Current evidence indicates that HIF-1α, and not HIF-2α, is active in regulating the transcription of genes that encode the enzymes that coordinate the activation of cellular pathways related to

ATP support[19]. HIF-1 is a major activator of pathways that play crucial roles in tumor development by upregulating pivotal genes. The genes thereafter regulate energy metabolism, angiogenesis, survival, invasion, metastasis, and drug resistance of cancer cells[20]. The HIF-1α subunit is regulated by O_2-dependent hydroxylation, ubiquitination, and degradation. In general, it is accepted that HIF signaling is activated in all types of cancer, including PC. Its activation will provide the tumor cells with tools to support survival. Therefore, HIF has been described as a key molecular target for cancer therapy. therapy[5,21]. To cite some examples, Xiao *et al*[22] showed that silencing the expression of HIF-1 diminished the invasiveness of Panc-1 and MiaPaca-2 cells. The enzyme prolyl hydroxylase domain 3 (PHD3) regulates the degradation of HIF and is the rate-limiting in that process. PHD3 is deregulated in PC cells. It has been shown that HIF-1α expression was suppressed in MiaPaca2 PC cells that overexpressed PHD3, which inhibited cell growth and colony formation under hypoxic conditions[19,23]. Another study showed that PHD3 expression regulated the secretion of VEGF evoked by hypoxia and inhibited tumor growth *in vivo* by abrogation of angiogenesis[24].

Along with cancer cells, the fibrotic tissue also undergoes adaptation to hypoxia by terms of HIF signaling. HIF-1α expression was found in the stroma adjacent to the PDAC cells[25]. HIF-1 and HIF-2 were expressed in cultured PSC subjected to hypoxia [14]. HIF-1 expression induced changes in PSC that promoted invasion and epithelial-mesenchymal transition and inhibited death of PC cells[22]. HIF-1α induced secretion of collagen in PSC. Accumulation of collagen together with other extracellular matrix components promotes fibrotic stroma formation[26]. Another study showed that HIF-1 α promoted the activation of PSC through recruitment of macrophages in PDAC[27].

The growth of fibroblasts was accelerated by the release of sonic hedgehog protein (SHH) in PC cells that were subjected to hypoxia, and that situation contributed to increased deposition of fibrous tissue[28]. PC stromal fibroblasts subjected to hypoxia were found to be associated with aggressive invasion and liver metastasis and increased processing and release of hepatocyte growth factor activator[29]. Moreover, fibroblasts have been found to contribute to vascular remodeling under hypoxia by influencing VEGF expression[30]. Figure 1 is a depiction of all the cell types that can be included in tumor tissue. Depending on their location in the mass, all cells are subjected to hypoxia to different extents.

OXIDATIVE STRESS AND ANTIOXIDANT RESPONSE UNDER HYPOXIA IN PC

Cancer cells have high proliferation rates, altered metabolism, and increased oxidative stress. The generation of reactive oxygen species (ROS) leads to genomic instability and the impairment of gene expression that underly cancer development[31]. ROS are produced in the mitochondria[32] and production is increased in tumor cells[6]. In healthy cells, uncontrolled overproduction of ROS is usually accompanied by cell death[33]. However, that does not necessarily happen in tumor cells, which can adapt to survive. Local expression of renin-angiotensin system has been signaled to play an important role in the regulation of blood pressure and fluid homeostasis in the pancreas[34]. Interestingly, hypoxia could upregulate the mentioned system that could then contribute to the modulation of local blood flow in a growing tumor and help the tissue to manage oxidative stress. Along this line, angiogenesis is a process that attempts to counter the lack of nutrients and oxygen shortage that develops in tumors [35]. However, in spite of the development of new microvasculature, vascular access is still poor and O_2 supply is compromised in the majority of tumors[36]. In PDAC for example, a low microvessel density and collapsed vasculature are observed[37].

To adapt to the adverse pro-oxidative conditions derived from uncontrolled cell proliferation and a limited blood supply, cancer cells increase their antioxidant defenses[38]. The redox signaling protein apurinic/apyrimidinic endonuclease 1/Redox effector factor 1 (APE1/Ref-1) modulates the redox activity of PC cells and is involved in cell proliferation and migration through the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and HIF-1 pathways[39]. The transcription factor nuclear factor erythroid-related factor 2 (Nrf2) regulates a wide range of cytoprotective genes in response to oxidative stress and various stressors present in the extracellular environment. The glutathione system is key among the antioxidant systems that are regulated by this transcription factor[40]. Chronic hypoxia is known to increase glutathione-dependent antioxidant capacity, which avoids damage to the cell membrane because of concomitant oxidative stress[41]. Indeed, glutathione

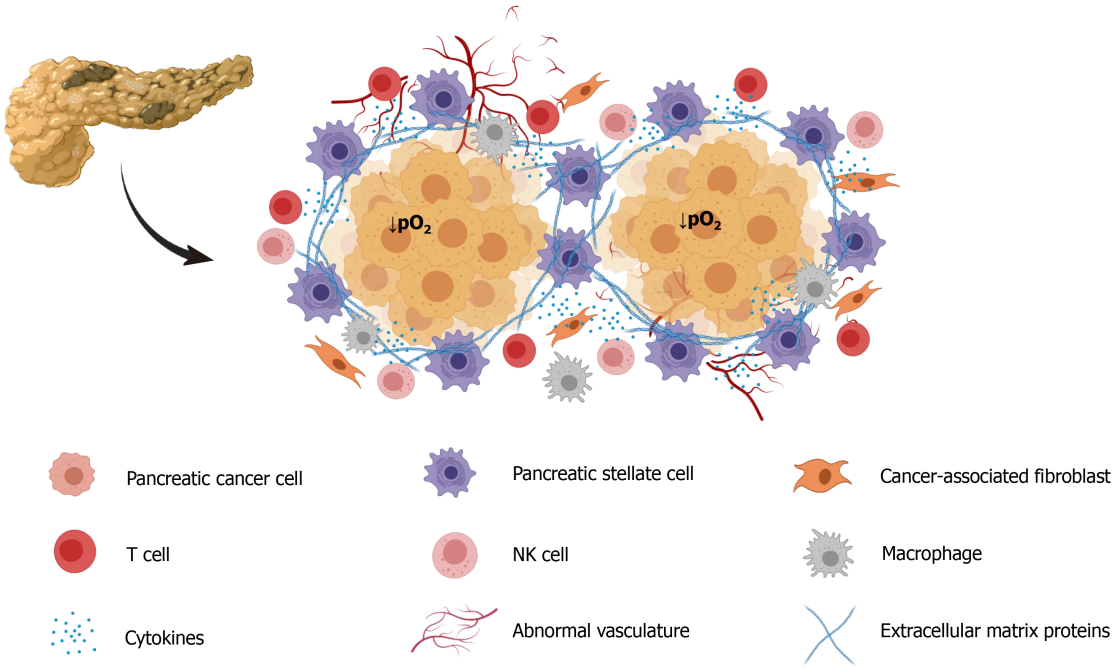


Figure 1 Illustration of the cells that form tumor tissue. Both tumor cells and the stroma contribute to the formation of tumor tissue. The latter comprises fibroblasts, stellate cells, immune cells, secreted components such as cytokines or extracellular matrix proteins, and the vasculature. Depending on their location in the mass, all cells are subjected to hypoxia to differing extents. Created with BioRender.com.

peroxidase maintains redox homeostasis in Panc1 cells, a pancreatic tumor cell line [42]. Heme oxygenase-1, an antioxidant enzyme regulated by Nrf2, has been shown to provide a survival advantage to PDAC cells subjected to hypoxia, and its inhibition induced an increase of the production of ROS and increased cell death[43]. Superoxide dismutases (SODs) are antioxidant enzymes that protect cells against ROS. The family consists of three isoforms, cytoplasmic Cu/ZnSOD (SOD1), mitochondrial MnSOD (SOD2), and extracellular Cu/ZnSOD (SOD3)[44]. A decrease in the expression of SOD1 has been related to a decrease in the viability of the pancreatic tumor cell lines Panc-1 and MiaPaCa2 cells when they were subjected to hypoxia[45]. SOD2 was shown to protect KP4 human pancreatic carcinoma cells against oxidative stress evoked by hypoxia/reoxygenation. The evidence thus supports the involvement of antioxidant enzymes in PC cell survival[6].

Interestingly, PSC undergo oxidative stress under hypoxia, and increased oxidation of lipids and proteins have been reported in PSC subjected to hypoxia. Moreover, PSC can adapt to hypoxia by increasing their antioxidant defenses by upregulated expression of SOD1 and SOD2 associated with increased phosphorylation of the Nrf2 transcription factor[14]. Figure 2 summarizes the involvement of the pathways involved in hypoxia-evoked antioxidant responses. In general, both the tumor cells and the other cells that grow in the mass adapt to varying extents. The overall success achieved by cellular responses determines the growth of the tumor and the probable concomitant migration, invasion, and metastasis[46].

HYPOXIA AND MITOGEN-ACTIVATED PROTEIN KINASE SIGNALING IN PC

Mitogen-activated protein kinases (MAPKs) comprise a family of proteins that regulate various physiological processes. Among them, cell proliferation, differentiation and survival play major roles in cancer development and progression[47]. Participation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and c-Jun amino-terminal kinases (JNK) in the growth of PC was demonstrated in KP-1N human pancreatic carcinoma cells that had been incubated with the secretagogue cholecystokinin[48]. Glucose deprivation, which was used to mimic hypoxia, induced the activation of JNK in the PC cells. Inhibition of the kinase led to a decrease of cell sensitivity to glucose deprivation-induced apoptosis[49]. JNK was also activated by

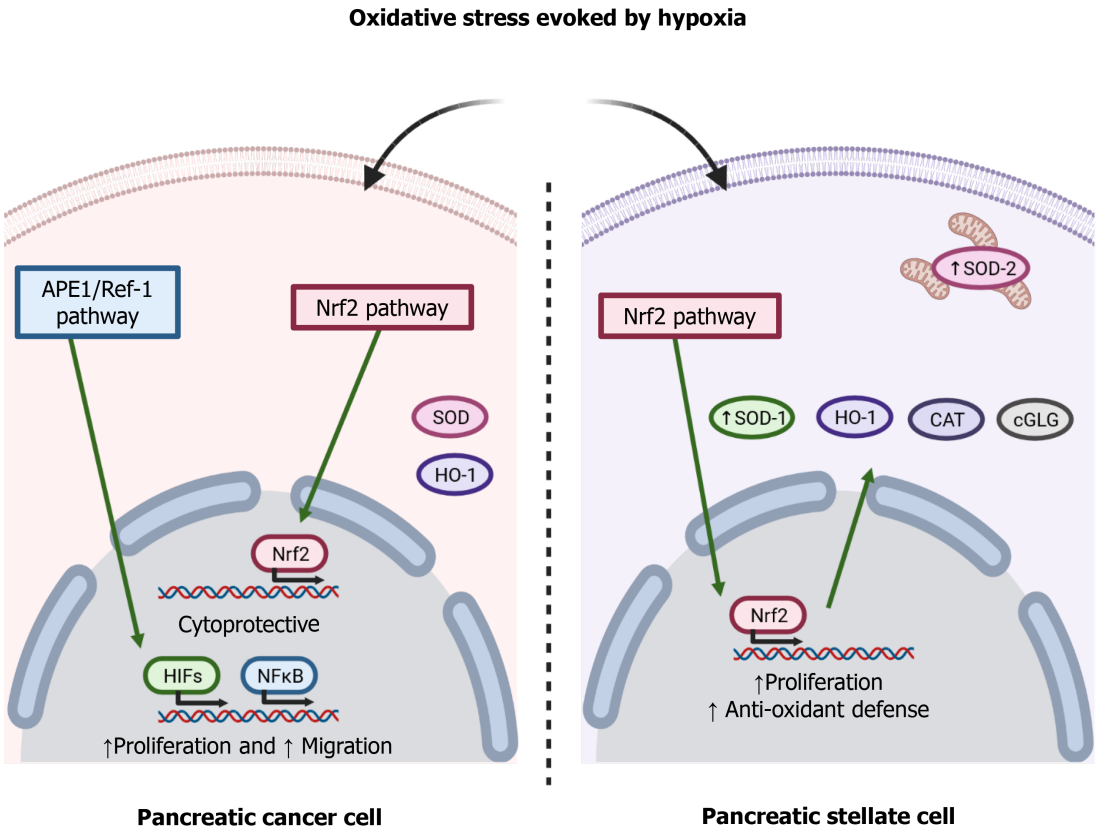


Figure 2 Hypoxia-evoked antioxidant response. In response to the increase in the generation of reactive oxygen species under hypoxia, cells activate intracellular pathways that provide antioxidant protection. Among them, the Nrf2 pathway plays a critical role by coordinating the activation of cytoprotective genes that lead to the expression of major antioxidant enzymes. Created with BioRender.com. APE1/Ref-1: AP endonuclease1/Redox effector factor 1; HIFs: Hypoxia-inducible factors; NF-B: Nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2: Nuclear factor erythroid-related factor 2; SOD: Superoxide dismutases.

interferon-induced protein with tetratricopeptide repeats 3, a group of genes that are stimulated by interferon and upregulated in aggressive PC cells[50]. Pro-survival and proliferation pathways regulated by ERK1/2 have been reported in Panc-1 cells[51]. In Capan-2 cells, another human PC cell line, hypoxia upregulated ERK1/2 and activated HIF-1α, which conferred chemoresistance of cells to gemcitabine[52]. On the contrary, a decrease in the phosphorylation of ERK1/2 was accompanied by a drop in the proliferation of L3.6pl human PC cells[53]. ERK1/2 were also involved in the malignant transformation and development of PDAC under hypoxia; and as has been shown in other pancreatic tumor cell lines, implication of HIF-1α expression was also reported [54].

Involvement of p38 MAPK in the modulation of cancer cell survival has also been shown. Its inhibition protected of MiaPaCa2 cells from death in response to 15-deoxy-delta-prostaglandin J2[55]. Conversely, p38 was activated in MiaPaCa2 cells subjected to simulated ischemia, and led to activation of HIF-1α[56]. Moreover, inhibition of p38 in MiaPaCa2 cells subjected to hypoxia-induced sensitization of cells to 2-deoxy-glucose and D-allose and decreased their viability. Involvement of HIF-1α was probably responsible for the antiproliferative activity because the inhibitor decreased HIF-1α protein accumulation and transcriptional activity[57]. Participation of MAPKs in the development of stromal tissue in cancer has also been proposed[58,59]. In a recent study it has been shown that PSC subjected to hypoxia exhibited an increase in the phosphorylation of JNK, whereas that of p44/42 and p38 was decreased. PSC survival under hypoxia was dependent on JNK activation because incubation of cells with the specific inhibitor SP600125 decreased cell viability[14]. Another member of the MAPK family, extracellular-signal-regulated kinase 5 (ERK5), is required for the prevention of apoptosis, regulation of hypoxia, tumor angiogenesis, and cell migration [60]. Inhibition of ERK5 diminished proliferation and migration of HepG2 and Huh-7 human hepatocellular carcinoma cells[61]. Implication of ERK5 in other types of cancer, including renal cancer[62], prostate cancer[63], and leukemia[64] has also been reported.

The ERK-5 inhibitor XMD8-92 inhibited pancreatic tumor growth *via* downregulation of doublecortin-like kinase 1 and several of its downstream targets that are involved in oncogenic pathways, including c-Myc, KRAS, NOTCH1, ZEB1, ZEB2, SNAIL, SLUG, OCT4, SOX2, NANOG, KLF4, LIN28, VEGFR1, and VEGFR2)[65]. Inhibition of p44/42 and ERK5 synergistically caused loss of Myc protein, which is a key transcriptional factor with a key role in cell growth, differentiation, and tumor development. Inhibition of both MAPKs suppressed PDAC growth[66]. However, additional studies on the participation of ERK5 in the development of PC are currently lacking.

To our knowledge, little is known about the involvement of MAPK signaling in PC under hypoxia because of a lack of published studies. The available evidence indicates that differential inhibition of protein kinases might be used to attenuate the progression of the disease, but it is not conclusive. Therefore, additional research should be conducted in to unravel how this important signaling pathway is affected by hypoxia and to elucidate whether the targeting of MAPKs could be used in cancer therapy. The putative involvement of MAPK signaling under hypoxia is summarized in Figure 3.

HYPOXIA AND APOPTOSIS IN PC

Apoptosis is a regulated cellular process that occurs both in physiological and pathological conditions. It is designed to control the cell population within a tissue or organ[67]. The pathways involved in apoptosis can undergo defects that might lead to the transformation of damaged cells. This commonly occurs in cancer and has been reported as responsible for development, growth, metastasis, and chemoresistance in different types of tumors[68]. Bcl-2 is a major protein in the apoptosis pathway. It is considered a gene that suppresses the pathway in the sense that overexpression of Bcl-2 blocks or delays the onset of apoptosis in cancer cells[69]; conversely, inhibition of Bcl-2 may lead cell death in some cancers that are resistant to apoptosis, for example breast cancer and PC[70]. Hypoxia has been associated with treatment resistance in various cancers, and apoptosis is among several signaling pathways that are thought to be involved. Many studies conducted in different cellular models of tumors have reported that the antitumor, and hence beneficial, effects of a variety of drugs depend on their ability to activate apoptosis[71]. However, few studies have focused on how the pathways that control apoptosis are modulated by hypoxia.

Cells with constitutive expression of HIF-1α have been shown to be more resistant to apoptosis than those without constitutive expression of the transcription factor, and the lack of expression was associated with increased *in vivo* tumorigenicity[72]. The expression of a regulator of hypoxia-induced cell death, Bcl-2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3), was downregulated in PC cells and was associated with adaptation of the tissue to hypoxia and tumor resistance. Restoration of BNIP3 expression increased cell sensitivity to death[73]. Decreased expression of thioredoxin 1 has been reported in human PC tissues and was associated with decreased apoptosis and increased cell survival. The induction of thioredoxin-interacting protein, a tumor suppressor gene, resulted in increased apoptosis of Panc-1 cells even in cells with activation of HIF-1α[74].

In a mouse pancreas endocrine tumor model, it was shown that increased invasion into surrounding exocrine tissue was associated with decreased the expression of caspase-3, and occurred in areas were high levels of HIF-1α were noted[75]. In a related study, cleaved caspase-3 staining was increased in viable tumor tissue of hypoxic models. That was considered as indicating a remodeling process of the growing tissue by a counterbalanced cell loss by apoptosis in response to rapid cell proliferation[76]. Another study showed that MiaPaca2 cells exhibited increased levels of caspase-3 when cells were transfected with HIF-1α siRNA. That resulted in a decrease of cell proliferation and in an increase of chemosensitivity[77]. Overexpression of HIF evoked the expression of hypoxia-induced gene domain family-1a (Higd-1a), a mitochondrial inner membrane protein. The gene expression was believed to promote cell survival under hypoxia, because apoptosis was decreased. The antiapoptotic effect of the genes resulted from the inhibition of cytochrome C release and from the reduction of caspase activity[78]. Another study showed that hypoxia and serum-free media, which were used to mimic tumor hypoxic-ischemic microenvironment, reduced apoptosis and stimulated proliferation of MiaPaCa2 cells[79].

With respect to the tumor microenvironment, it is well accepted that it contributes to resistance to cancer therapy and to the evolution of the disease by regulation of

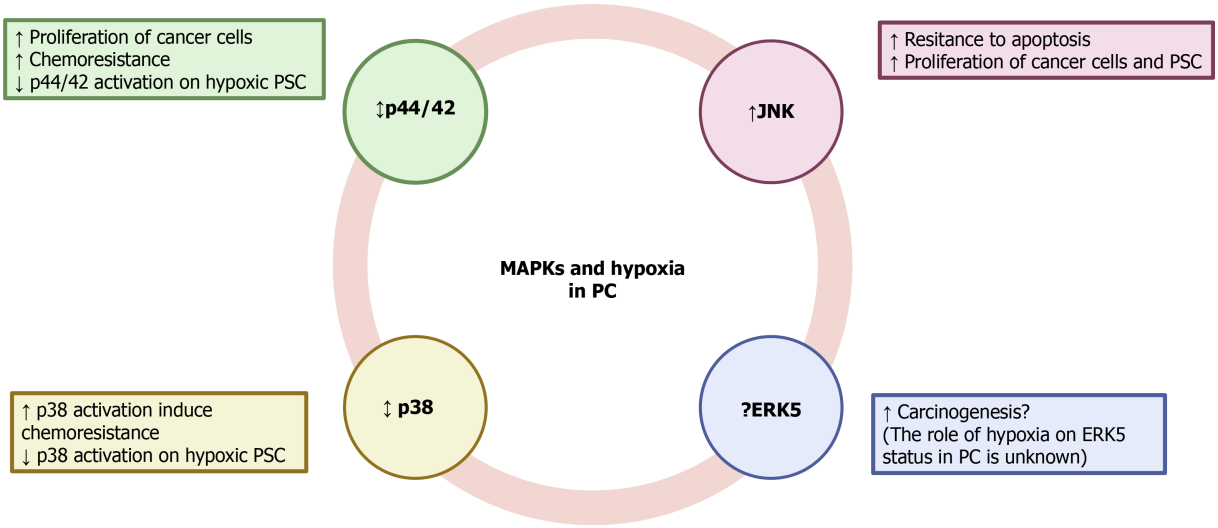


Figure 3 Contribution of mitogen-activated protein kinase signaling to cell responses under hypoxia. Mitogen-activated protein kinases comprise of a family of proteins that regulate physiological processes such as cell proliferation, differentiation, and survival, which play major roles in cancer development and progression. Differential inhibition of protein kinases might be used to attenuate the progression of the disease. Created with BioRender.com. ERK5: Extracellular-signal-regulated kinase 5; JNK: c-Jun amino-terminal kinase; MAPKs: Mitogen-activated protein kinases; PC: Pancreatic cancer; PSC: Pancreatic stellate cells.

apoptosis[80]. Hypoxia has been shown to regulate the viability of PSC through p21-activated kinase 1. The inhibition of this kinase decreased the activation, inhibited proliferation, and increased apoptosis of human PSC[12]. TH-302, a hypoxia-activated prodrug, was assayed in MiaPaCa2 and PANC-1 cell lines, and in BxPC-3 PDAC xenograft models in mice. The compound helped to reduce stromal density and intratumoral hypoxia, and hence, increased the effectiveness of anticancer drugs[81]. The expression of fibulin-5 (Fbln5) is abundant in the stroma of PDAC[82]. This matricellular protein supports PDAC progression by blocking fibronectin-integrin interaction. Compared with normal pancreatic tissue, Fbln5 content was increased in mouse embryonic fibroblasts and 3T3 fibroblasts subjected to hypoxia. This involved transforming growth factor β (TGF-β)- and PI3K-dependent mechanisms, because inhibition blocked hypoxia-induced Fbln5 expression[83]. The balancing of apoptosis pathways could be a maneuver of PC cells to support the growth of tumor tissue[84]. Moreover, the modulation of HIF expression might be a valuable tool to control tumor cell proliferation under hypoxia[85].

AUTOPHAGY AND ADAPTATIONS OF CELLULAR BIOENERGETICS UNDER HYPOXIA IN PC

Autophagy is a metabolic pathway that is used by the cell for degradation of cytoplasmic proteins, macromolecules, and organelles in the lysosomes. It serves as a mechanism for protection of the cell and represents a major survival pathway that is activated in the presence of environmental and cellular stress[86]. However, is relationship to cancer development is contradictory. Both cell survival and cell death have been associated with autophagy because defects or partial reduction in autophagy transmit oncogenic stimulus and have been associated with cancer cell survival[70]. Autophagy is also activated under hypoxia and has been reported as potential contributor to the resistance of various types of cancer to therapy[71].

Inhibition of the pAkt/mTORC1 pathway, one of the critical regulators of autophagy, was observed in PC cells incubated under hypoxia, was found to promote autophagy, and was associated with enhanced cell survival[87]. Autophagy induction in response to hypoxia was detected in the PANC-1, BxPC-3 and AsPC-1 pancreatic cell lines, in which inhibition of AMP-activated protein kinase induced cytotoxicity and enhanced apoptosis. This suggested the involvement of this pathway in cell survival under hypoxia[88]. Serum-free media and hypoxic conditions protected MiaPaCa2 cells against death by stimulating autophagy. In that study, the ratio of LC3-II to LC3-I expression was increased, and treatment with the inhibitor of autophagy 3-MA decreased cell viability[79]. Autophagy was inhibited by chloroquine in MiaPaCa2 and S2VP10 cells that had been subjected to hypoxia, suggesting that the

pathway was responsible for tumor cell survival[89]. Autophagy was also involved in the survival of PDAC cells in spite of the deprivation of nutrients and O_2 that derived from the hypoxic tumor microenvironment[90]. In the aforementioned study, LC3 was converted to the active LC3-II form, formation of autophagic and acid vesicles was detected, and PC cell survival and migration increased. Similarly, intermittent hypoxia increased the levels of HIF-1α. Enhanced autophagy was associated with increases in the levels of LC3-II and Beclin. As a consequence, hypoxia-induced stem-like properties of non-stem PC cells, which influenced tumor development and growth [91]. Analysis of the expression of LC3 in different PC tissue samples revealed strong LC3 expression in the peripheral areas, which was related to poor outcome. It was concluded that activation of autophagy was associated with the responses of cells to factors in the cancer microenvironment, including hypoxia[92].

Changes in metabolism related to mitochondrial and bioenergetics adaptations also occur in response to hypoxia. It has been demonstrated that tumor cells survive and proliferate under hypoxic and glucose-deprived conditions that develop in PCs that are caused by a lack of vasculature that leads to a low blood supply to the growing tissue and to changes in energy metabolism[49]. In the same study 63 genes were identified, whose expression was increased under conditions that mimicked hypoxia. Thus, the expression of certain genes may determine the survival of cancer cells when the blood supply of O_2 is compromised[49]. Mitochondria are the major organelles responsible for supplying energy to the cell in the presence of O_2[93]. It has been suggested that mitochondria undergo changes in dynamics and distribution that are an adaptive response for survival and metastasis[94]. Along this line, PDAC cell lines are able to grow even when O_2 tension is as low as 0.1%. Under such conditions, the cells maintain the mitochondrial parameters and oxidative metabolism needed for the synthesis of metabolites that maintain proliferation[8].

Glycolysis is of major relevance to tumor survival and proliferation because is the main provider of energetic substrates[95]. MUC1 is a large type I transmembrane protein that is overexpressed in several carcinomas including PDAC. It regulates, through interaction with HIF-1α, the expression of genes involved in glucose metabolism and enhances glycolytic activity[96]. Hypoxia has been shown to induce adaptive metabolic responses, such as a high glycolytic rate and activation of the hexosamine biosynthetic pathway, which favor hypoxic and normoxic cancer cell survival[97]. Hypoxic glycolysis, with increases in glucose uptake and lactate production, was found to be governed by ERK2 in PDAC cells and was associated with malignant progression[54]. Phosphoglycerate kinase 1, an enzyme that is involved in the generation of ATP in glycolysis, is involved in the energy supply in PDAC cells *via* Nuclear Factor of Activated T Cells 5[98]. Activation of glycolysis was shown in another study carried out using PDAC cells subjected to hypoxia. The authors showed that carbonic anhydrase 9 (CA9), an enzyme that is involved in the regulation of cellular pH, was upregulated. It functions as a part of the cellular response to hypoxia to promote cell survival. The inhibition of CA9 reduced the cellular pH, decreased glycolysis, and increased cellular sensitivity to gemcitabine[99]. The use of inhibitors of CA9 was also suggested by Logsdon *et al*[100], who showed that inhibition of APE1/Ref-1 decreased HIF1α-mediated induction of CA9 and might reduce PDAC cells viability.

The expression of the enzymes 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 and -4 (PFKFB-3 and PFKFB-4) was increased in different cancer cell lines, including PC, in response to HIF[101]. It is known that those enzymes play a major role in the regulation of glycolysis in cancer cells to support proliferation and survival. Correlation with the enhanced expression of VEGF and glucose transporter-1 (Glut-1) was observed[101]. The M2 isoform of pyruvate kinase (PKM2), a molecule involved in glycolysis, was shown to participate in the metabolic reprogramming of cancer cells and regulated cell cycle progression. Impairment of PKM2 expression decreased cell proliferation and augmented apoptosis and, hence, impaired tumor growth[102]. Another study showed that the activity of aldehyde dehydrogenase 1 was increased in cells subjected to hypoxia and was associated with the induction of colony and spheroid formation[103].

Glucose enters the cell *via* glucose transporters (GLUTs), of which various classes exist[104]. Targeting GLUTs might be a valuable tool to help anticancer drugs to exert their effects[105]. Increased expression of Glut-1 in cancer cells can maintain the energy supply needed for development and growth[106]. Expression of the genes Glut-1 and aldolase A, which are associated with the regulation of anaerobic metabolism, was found to be increased in cells with constitutive expression of HIF-1α compared with cells without it. It was assumed that expression of HIF-1α was associated with increased survival and proliferation of PC cells, and that cells undergo

activation of anaerobic metabolism under conditions of hypoxia and glucose deprivation[72].

Adaptation responses to glucose deprivation and hypoxia are also necessary for the survival of cells in the tumor microenvironment[107]. A number of genes, including Glut-1, Glut-3, and Hexokinase-2, were expressed at high levels in human PC tissue specimens where hypoxic conditions were detected[108]. Guillaumond *et al*[97] reported that hypoxic PDAC tissue was comprised of abundant epithelial cells harboring epithelial-mesenchymal transition features and expressing glycolytic markers. Hypoxia led to a change in the glycolytic metabolism of PC cells from oxidative phosphorylation to lactate production. As a consequence, the growth of normoxic cancer cells was favored[109]. Interestingly, PDAC cells might use PSC-derived alanine to increase their biochemical flexibility in order to adapt to the austere conditions resulting from hypoxia[110]. Finally, Munc18-1-interacting protein 3 (Mint3) was found to promote ATP production *via* glycolysis by activating HIF-1α in cancer fibroblasts[58].

HYPOXIA AND ENDOPLASMIC RETICULUM STRESS IN PC

Specific conditions and factors within tumor masses, for example hypoxia, nutrient starvation, low pH, and increased levels of free radicals, induce a situation that has been termed "endoplasmic reticulum (ER) stress". It is also known as unfolded protein response[111]. In tumors, this response is found in malignant cells and other cell types present in the stroma[112]. In general, ER stress works in favor of cell survival and adaptation to hostile environmental conditions. Nevertheless, the ER stress can also induce cell death if it is unresolved. It can also be connected to inflammation and immune suppression within tumors[113]. For information about the branches operating ER stress see Chern and Tai[114].

As mentioned above, glucose deprivation and hypoxia often occur in solid tumor cells, including PC cells. A study showed that with glucose deprivation, ER stress was activated in cancer cells and upregulated glucose-regulated protein 78 (GRP78), which protects cells from death. Inhibition of ER stress by pancastatin A (PST-A) and B (PST-B), which are glabretal triterpenoid moieties, suppressed the accumulation of GRP78, and exhibited selective cytotoxicity in Panc-1 cells[115]. Hypoxia and ER stress contributed to the overexpression of endoplasmic reticulum oxidoreductase 1 alpha (ERO1L) in PDAC. ERO1L is an ER luminal glycoprotein that participates in the formation of disulfide bonds in secreted and membrane proteins. This oxidoreductase has been associated with the proliferation of PDAC cells *in vitro*[116]. To our knowledge, little is known about how ER stress evolves under hypoxia. Further studies need to be carried out in order to better understand how this dual-sided pathway determines cell fate under hypoxia.

HYPOXIA AND INFLAMMATION IN PC

PDAC is characterized by a significant inflammatory response[103]. During malignant progression, cancer cells acquire various features that include increased secretion of VEGF and interleukin 6 (IL-6), to which inflammation contributes to a certain extent and confers chemoresistance[50]. Hypoxia induces the accumulation of metabolites that can enter signaling cascades and contribute to the inflammatory response[117]. The expression of IL-6 was enhanced by hypoxia and proteins involved in PC development like Kras, mesothelin or ZIP4. The cytokine contributed to the generation of a pro-tumorigenic microenvironment and was probably involved in angiogenesis [118]. The expression of IL-6 was also reported as responsible for the aggressiveness of PC cells under hypoxia[119]. PC cell lines expressed higher levels of IL-6 than normal human pancreatic tissue. Moreover, exogenous application of IL-6 in Panc-1, MIA PaCa-2, and BxPC-3 cells increased the secretion of multiple Th2-type cytokines. In addition, IL-6 activated ERK2 signaling pathways[120]. Under hypoxia, involvement of downstream elements of the pathways regulated by VEGF and tumor necrosis factor has been suggested[121].

Human IL-37 possesses anti-inflammatory and immunosuppressive properties. It also suppresses tumor growth and progression[122]. A study by Zhao *et al*[123] showed that HIF-1α attenuated IL-37 transcription. A decrease in the expression of IL-37 was observed in PDAC this was associated with increased histological grade, tumor size, metastasis, and vessel invasion. On the other hand, IL-37 suppressed the

expression of HIF-1α through the inhibition of Stat3. Stat3 exhibits a range of oncogenic functions that include suppression of antitumor immune responses and promotion of inflammation. Another found showed that PC cell death was induced by blockade of the expression of Stat-3-related genes[124]. It has been suggested that IL-1β was involved in cell proliferation after Myc-induced cell cycle entry. Following Myc activation, cells rapidly exhibited expression and release of the proinflammatory cytokine IL-1β, which could be the reason why pleiotropic Myc oncoproteins contributed to the expansion of the vascular compartment during tumor progression [124].

In PDAC the abundant stroma participates in the recruitment of immunosuppressive cells through the activation of cancer-associated fibroblasts and the secretion of TGF-β[125]. It has been proposed that PSC and myofibroblasts subjected to hypoxia contribute to the recruitment of myeloid-derived suppressor cells and B cells in PDA caused by the release of cytokines[26]. Recruitment of macrophages in PDAC was found to induce the activation of PSC that was associated with a certain degree of inflammation in the tumor region. The process was dependent on HIF-1α[27]. In a study by Maruggi *et al*[126], it was shown that hypoxic cancer cells secreted the inflammatory chemokines IL-1β and IL-8. Moreover, analysis of the tumor stroma revealed enrichment of myeloid dendritic cells 1 and 2, which secreted proangiogenic cytokines. Another study showed that IL-8 was significantly overexpressed in regions surrounding necrotic areas of PC tissue in which the cells were exposed to hypoxia and an acidic pH. IL-8 was reported to participate in the growth and metastasis of variants derived from COLO 357 human PC cells[127]. Additionally, IL-8 increased tumorigenesis by promoting angiogenesis and metastasis *via* VEGF and neuropilin expression. IL-8 also stimulated ERK1/2 signaling in the human PC cell line BxPC-3 [128]. It may be possible that controlling the inflammatory response and the release of cytokines by cells within the tumor mass would facilitate treatment and restrain tumor growth[129].

EXTRACELLULAR VESICLES AND HYPOXIA IN PC

Extracellular vesicles, also known as exosomes, consist of diverse types of membrane vesicles of endosomal and plasma membrane origin that are released by the cells into the extracellular milieu[130]. They are considered as an important mode of cell-to-cell communication because they function as vehicles to transfer membrane and cytosolic proteins, lipids, and RNA between cells[131]. The hypoxic microenvironment promotes tumor cells to release exosomes, which is an activity of solid tumors that allows invasion and metastasis[132]. As in other types of cancer, exosomes have a key role pancreatic tumor pathobiology by facilitating intercellular communication. Exosome, are considered as important mediators of the crosstalk between tumor cells and the microenvironment[133].

Hypoxia was found to stimulate the release of exosomes in MiaPaCa2 and AsPC1 cells and to promote the survival of cells subjected to hypoxia. HIF-1α was involved in exosome release[134]. Another study showed that exosomes derived from PC cells subjected to hypoxia-activated macrophages, a process that was dependent on HIF-1α or HIF-2α. Following release, the exosomes facilitated the migration, invasion, and epithelial-mesenchymal transition of PC cells, thereby worsening the prognosis[133]. Regarding the stroma, feedback loops are established between stromal elements and tumor cells in the cancerous tissue. Along this line, PSC are responsible for direct nutrient transfer *via* vesicles[135]. As with other aspects that have been reviewed above, modulation of exosome signaling under hypoxia deserves further study.

MICRORNA AND HYPOXIA IN PC

Micro (mi)RNAs are noncoding RNAs comprising of a single-stranded chain of 18 to 22 nucleotides, and they play major roles in the regulation of gene expression. Extracellular miRNAs function as chemical messengers to mediate cell-to-cell communication[136]. Synthesized miRNAs can be released from the cell into the extracellular space by (1) selective incorporation in the exosomes, (2) being coupled with Ago2 protein, and (3) by release attached to high-density lipoproteins. Once in the extracellular medium miRNAs will reach other cells to alter their functions[133, 137].

Hypoxia was found to decrease the expression of miR-519 in Panc-1 and SW1990 cells[138], and was regarded as a survival mechanism because transfection with miR-519 mimics inhibited cell growth and invasiveness and induced apoptosis. Similarly, HIF-1α induced the downregulation of miR-548an in PC cells during hypoxia. The vimentin content was inversely correlated with miR-548an expression and was associated with facilitation of pancreatic tumorigenesis[139]. miR-454 was found to be present in low levels in PDAC and it was associated with cell growth because overexpression of the miRNA led to slower cell growth[140]. The expression of other miRNAs has been associated with increased cell proliferation and cancer malignancy [141]. miR-210, which is induced by HIF-1α, was reported to have many targets within cells that can regulate the cell cycle, mitochondrial oxidative metabolism, angiogenesis, DNA damage response, and thus, cell survival[142]. A regulatory mechanism of HIF-1α on miR-191 expression was detected in MiaPaCa2 and Aspac1 cells subjected to hypoxic conditions. The expression of miR-191 in tissues from PC patients was closely associated with tumor size, stage, lymph node metastasis, and perineural invasion [143]. Similarly, miR-212 was associated with increased tumor size, lymph node metastasis, and vessel invasion in PDAC. A positive correlation between this miRNA and the expression of HIF-1α was noted, indicating that the miRNA was induced by hypoxia[144]. Hypoxia also increased the detection of miR-21 in BxPC-3 and AsPC-1 PC cells. Antisense constructs targeting miR-21 diminished cell proliferation by activating apoptosis[145]. Similarly, a study by Bao *et al*[119] reported that miR-21 and miR-210 were associated with increased cell migration, invasion, and angiogenesis.

The long noncoding (lnc)RNA UCA1 released from hypoxic PC cells and was shown to promote angiogenesis and tumor growth both *in vitro* and *in vivo* by regulation of miR-96-5p[132]. The synthesis and release of circ_0000977 was induced by hypoxia and conferred resistance to natural killer (NK) cells by Panc-1 cells. Inhibition of miR-153-mediated repression of HIF-1α and ADAM10 allowed the immune escape of the cancer cells[146].

Another hypoxia-induced microRNA, miR-646, blocked the expression of migration and invasion inhibitory protein (MIIP), which has been identified as an inhibitor of tumor development. MIIP acted to suppress the activity of histone deacetylase 6 and promote the degradation of HIF-1α, therefore impairing HIF-1α accumulation[147]. Inhibition of MIIP by miR-646 led to increased proliferation and invasion of PC cells. miR125a, the content of which was regulated by HIF-1α, inhibited mitochondrial fission and contributed to cellular survival in PDAC by preventing apoptosis[148].

The existing evidence signals that miRNAs differentially participate in regulating cell fate. Whereas certain types are downregulated in cancer cells, others have increased expression compared with healthy cells[149,150]. The expression of miRNAs is regulated by HIF-1α when hypoxic conditions are established, and that confers the tumor cells with adaptations that allow cancer growth. A summary of the roles of miRNAs in PC under hypoxia is provided in Table 1. Fewer studies have investigated miRNAs in the pancreatic stroma. Along this line, Bynigeri *et al*[151] reported that miRNAs were involved in the inflammatory and profibrogenic functions of PSC. Within the stroma, the interaction of PSC and tumor cells that include signaling through miRNAs can promote tumor progression, metastasis, immune evasion, and drug resistance, which impact the evolution and prognosis of PC[152].

POTENTIAL TREATMENTS DIRECTED AGAINST HYPOXIA

As noted earlier in this review, cells growing within the tumor mass make key adaptions and changes in intracellular pathways that might convey resistance to death and to treatment. In fact, the most frequent treatment of PDAC is radical surgery and chemotherapy, and they are the only treatments that seems to work in a only a few patients. Despite treatment, PDAC remains a highly lethal disease. In addition to surgery, various combinations of drugs of different types have been evaluated as chemotherapy and remains as the standard adjuvant therapy[153]. For example, the combination of FOLFIRINOX and nab-paclitaxel plus gemcitabine as found to improve the survival of patients with metastatic disease[154] and to improve surgical and clinicopathologic outcomes following pancreatic resection[155].

It must be noted that failure of chemotherapy, targeted therapy, and immunotherapy have all been attributed to the PDAC microenvironment[156]. Dysregulation of the tumor microenvironment promotes an intense fibrosis and immune suppression that plays a key role in drug resistance[157]. Therefore, targeting the extracellular tumor microenvironment *via* inhibition of signaling pathways, alteration of DNA

Table 1 List of oncogenic and tumor suppressor microRNAs in pancreatic cancer cells under hypoxia

MicroRNA	Oncogenic activity	Function	Ref.
miR-519	Anti-tumor	Inhibits cell growth and invasiveness	[138]
miR-58an	Anti-tumor	Reduces tumorigenesis	[139]
miR-454	Anti-tumor	Decreases cell proliferation	[140]
miR-210	Pro-tumor	Regulates cell cycle and promotes angiogenesis	[142]
miR-191	Pro-tumor	Promotes tumor growth, invasion, and metastasis	[143]
miR-212	Pro-tumor	Promotes tumor growth, vessel invasion, and metastasis	[144]
miR-21	Pro-tumor	Induces cell proliferation, invasion, and metastasis	[119,145]
miR-210	Pro-tumor	Increases migration, invasion, and angiogenesis	[119]
miR-646	Pro-tumor	Promotes proliferation and invasion	[147]
miR-125a	Pro-tumor	Increases tumor cell viability	[148]
miR-96-5p	Anti-tumor	Inhibits angiogenesis	[132]
miR-153	Anti-tumor	Avoids immune escape of tumor cells from natural killer cells	[146]

repair pathways, immunotherapy and/or modulation of cell metabolism, might serve as novel tools for PDAC treatment. Treatments under study include vaccines, oncolytic viruses, MEK inhibitors, cytokine inhibitors, and targeting hypoxia[125]. Recently, intense efforts have been carried out to evaluate the effectiveness of therapies aiming to increase tumor immunogenicity and promote the recruitment and activation of effector T cells[125]. Along this line, it has been suggested that challenging the immunosuppression occurring within pancreatic immune infiltrate might diminish tumor aggressiveness[154]. Because hypoxia contributes to aggressive tumor behavior, mainly involving tumor progression, malignancy, and promoting resistance to conventional and targeted therapeutic agents[158], finding drugs that are effective in the modulation of cell proliferation under hypoxia is a major challenge. HIF has been seen as a potential therapeutic target in the pathobiology of PDAC. Interestingly, HIF might exert its oncogenic influence through the modulation of the stroma rather than the modulation of cancer cells[159]. Cyclopamine, a hedgehog inhibitor plus paclitaxel in a polymeric micelle formulation, exhibited effects on the stroma by increasing microvessel density, alleviating hypoxia, and reducing matrix stiffness while maintaining the tumor-restraining function of extracellular matrix. As a result, tumor growth was suppressed and animal survival was prolonged[160]. Targeting HIF by siRNAs delivered to cancer cells might be an effective cancer treatment. Indeed, recombinant adeno-associated virus has been employed to deliver siRNA targeting HIF-1α into MiaPaCa2 human PC cells subjected to hypoxia. As a consequence, cell proliferation and migration decreased and apoptosis was induced [85]. siRNA targeting of HIF-1α in MiaPaCa2 cells subjected to hypoxia-induced apoptosis through both NF-kB-independent and -dependent mechanisms[161].

Additionally, hypoxia-activated prodrugs designed to overcome the resistance of cancer cells have shown clinical efficacy[162]. TH-302 is an investigational hypoxia-activated prodrug. Its combination with gemcitabine and nab-paclitaxel was effective in PDAC xenograft models in mice[81]. Moreover, TH-302 significantly decreased *in vivo* tumor growth, increased survival in a MiaPaCa cancer model and improved survival in Hs766t tumors[162]. Recently, we showed that PSC adapted to pro-oxidant conditions under hypoxia, and that the adaptations may have been responsible for increased cell viability and proliferation[14]. Interestingly, melatonin modulated the antioxidant responses and signaling by inflammatory regulators. Therefore, this indoleamine is emerging as another potential treatment of PDAC[163].

CONCLUSION

Hypoxia is a common condition that is created in solid tumors and it results from rapid, uncontrolled growth of the tissue that is accompanied by insufficient development of blood microcirculation. Along with tumor cell proliferation, a fibrotic reaction is established that involves the parallel growth of several types of cells

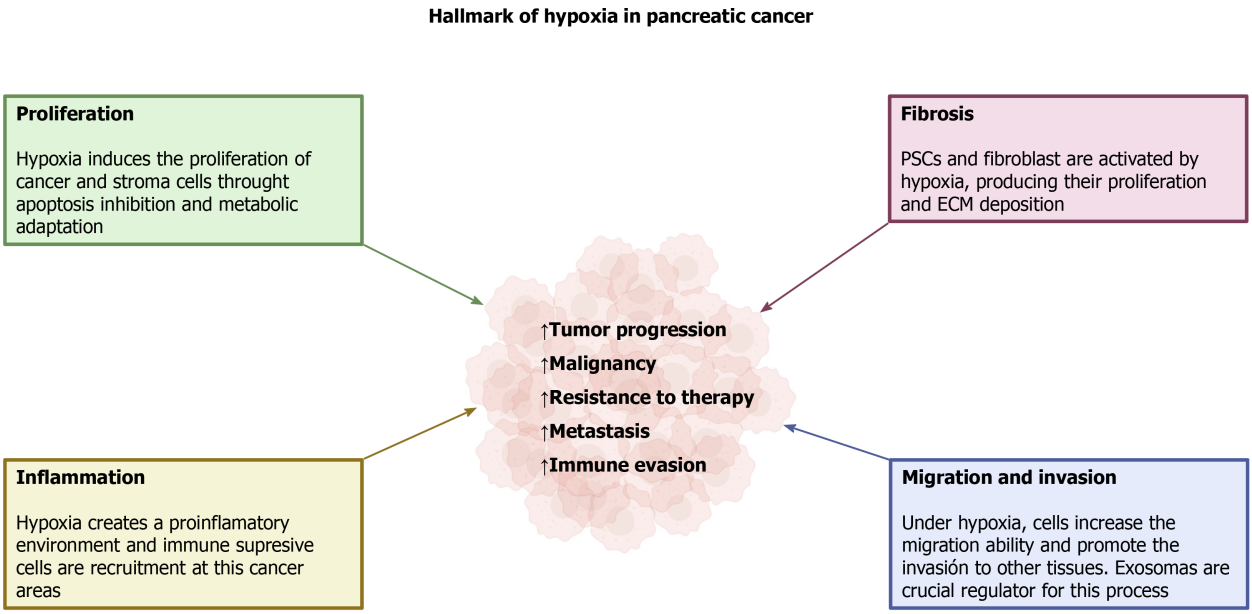


Figure 4 Hallmarks of hypoxia in pancreatic cancer. The figure summarizes the major adaptive features of pancreatic tumor cells in response to hypoxia. In general, low O_2 availability worsens the prognosis of pancreatic cancer by promoting increases in tumor progression, drug resistance, immune evasion, and metastatic ability. Created with BioRender.com. ECM: Extracellular matrix; PSC: Pancreatic stellate cells.

different from tumor cells, and the deposition of extracellular components, which comprises a stromal component. As a consequence of the low O_2 availability that develops, all the cells included in the mass adapt in order to survive. HIF is the major regulator of cell responses to hypoxia. Activation of different intracellular pathways and changes in cellular energy metabolism take place, and are all modulated by hypoxia. These conditions are responsible for tumor progression, malignancy, and resistance to therapy, which are major features of PC. Additionally, capabilities of invasion of neighbor tissues, organs, and metastasis are acquired, all of which are critical determinants of the extreme malignancy of PC. A summary of the major adaptations of pancreatic tumor to hypoxia is shown in Figure 4.

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REVIEW

Viral hepatitis: Milestones, unresolved issues, and future goals

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Abstract

In this review the current overall knowledge on hepatitis A, B, C, D, and E will be discussed. These diseases are all characterized by liver inflammation but have significant differences in distribution, transmission routes, and outcomes. Hepatitis B virus and hepatitis C virus are transmitted by exposure to infected blood, and in addition to acute infection, they can cause chronic hepatitis, which in turn can evolve into cirrhosis. It is estimated that more than 300 million people suffer from chronic hepatitis B or C worldwide. Hepatitis D virus, which is also transmitted by blood, only affects hepatitis B virus infected people, and this dual infection results in worse liver-related outcomes. Hepatitis A and E spread *via* the fecal–oral route, which corresponds mainly to the ingestion of food or water contaminated with infected stools. However, in developed countries hepatitis E is predominantly a zoonosis. Although hepatitis A virus and hepatitis E virus are usually responsible for a self-limiting hepatitis, a serious, rarely fatal illness is also possible, and in immunosuppressed patients, such as organ transplant recipients, hepatitis E virus infection can become chronic. The description of goals achieved, unresolved issues, and the latest research on this topic may make it possible to speculate on future scenarios in the world of viral hepatitis.

Key Words: Viral hepatitis; Hepatitis A; Hepatitis B; Hepatitis C; Hepatitis D; Hepatitis E

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Core Tip: Viral hepatitis still endangers the health of millions of people around the world. In this review the path that led to the current knowledge in the field of viral hepatitis will be reported.

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INTRODUCTION

Hepatitis viruses are a leading cause of morbidity and mortality as the consequences of their acute or chronic infections. The World Health Organization (WHO) estimated 1.4 million deaths per year due to viral hepatitis worldwide, most of which are a consequence of hepatitis B or C. Significant progress has been made in the prevention and treatment of hepatitis B, C, and D viruses (HBV, HCV, and HDV) infections, but chronic hepatitis by these viruses still represents the most common etiology of cirrhosis and hepatocellular carcinoma (HCC). Hepatitis A virus (HAV), HBV, HDV, and hepatitis E virus (HEV) are a frequent cause of clinical hepatitis and acute liver failure. Although it is rare, the latter is a life-threatening condition. In 2016, the WHO Global Health Sector Strategy established the goal of eliminating viral hepatitis "as a major public health threat" by 2030. However, great efforts are still needed to bridge the gap between the current situation and this ambitious goal.

HEPATITIS C

HCV is a single strand positive sense RNA virus of the Flaviviridae family (Hepacivirus genus) discovered in 1989 by Michael Houghton and colleagues[1,2]. This discovery, which was awarded the 2020 Nobel Prize in Physiology or Medicine, shed light on blood-transmitted chronic non-A, non-B hepatitis cases, and through the subsequent advent of specific therapies is estimated to have saved millions of lives. HCV is blood-borne transmitted[3], and currently eight viral genotypes are recognized [4,5]. In 15% of cases HCV infection is responsible for acute hepatitis[6], while approximately 80% of infected patients progress to chronic infection; 20% of chronically infected patients develop cirrhosis within 25 years, and among patients with cirrhosis 25% develop HCC and/or decompensated liver disease[7].

EPIDEMIOLOGY

HCV is widespread all over the world, with different prevalence of viral genotypes within different geographical areas. Genotype 1 is the most common, especially in North America, Europe, and Asia. Genotype 2 is endemic in parts of West Africa and Latin America. Genotype 3 is present in Southeast Asia and Eastern Europe. Genotype 4 is endemic in North Africa and Sub-Saharan Africa. Genotype 5 is particularly common in Southern Africa[8]. This virus, whose only host is humans, is transmitted by blood. In high-income countries this infection occurs particularly in "at risk" populations, such as people who inject drugs who may share infected needles, men who have sex with other men, and prisoners. In low-middle-income countries, healthcare associated transmission (*e.g.*, during invasive procedures or the execution of blood transfusions with infected blood) is still an important problem[9,10]. Perinatal transmission from infected mothers occurs in 3%-10% of cases[11].

CLINICAL MANIFESTATION

Acute HCV infection is usually asymptomatic and characterized by increased transaminases usually occurring between 6 wk and 8 wk after exposure[12]. Symptoms such as nausea, asthenia, and weight loss appear in less than 30% of patients, and in most cases they do not interfere with normal daily activities[13]. The presence of jaundice is also infrequent, occurring in less than one third of patients[14]. Acute liver failure due to HCV infection is unusual[12].

About 30% of people who contract the infection spontaneously eliminate the HCV within 6 mo. On the contrary, at least 60% to 70% of infected people develop chronic infection, defined as the presence of anti-HCV immunoglobulin in the blood and the persistence of HCV-RNA for at least 6 mo[12,15]. In these cases, transaminases are

usually moderately increased but can also be normal[16]. Most patients with chronic hepatitis C are asymptomatic, but some may experience asthenia, anorexia, and weight loss. Jaundice occurs only in the case of advanced liver cirrhosis. Physical examination can reveal hepatomegaly and splenomegaly (this latter in case of cirrhosis or blood malignancies)[12]. Chronic HCV infection could induce liver fibrosis, which can progress to the development of cirrhosis, decompensation, and HCC[17]. In HCV patients, HCC usually appears in patients with liver cirrhosis, but it can also occur in the absence of such condition[12]. The proportion of patients who develop liver cirrhosis after 20 years of infection is quite varied, ranging between 2%-3%[18,19] and 51%[20], depending on the study population and various risk factors, such as male gender, alcohol consumption, older age at the time of infection, coinfection with other viruses [human immunodeficiency virus (HIV) and HBV], and others[7].

EXTRAHEPATIC MANIFESTATIONS

The HCV primarily affects the liver, but a high percentage of HCV patients (up to 74%) was observed to have at least one extrahepatic manifestation[21]. For some extrahepatic manifestations the link with HCV was strongly demonstrated by epidemiological and pathogenetic data (mixed cryoglobulinemia/cryoglobulinemic vasculitis and B-cell non-Hodgkin's lymphoma), while others have a less close connection with this virus[22,23]. Mixed cryoglobulinemia is a small vessel vasculitis that predominantly affects the skin, joints, peripheral nerves, and kidneys, and in 80% of cases is caused by HCV. Clinical manifestations are variable, ranging from mild manifestations (purpura or arthralgia) to more serious conditions such as glomerulonephritis or generalized vasculitis[24]. B-cell non-Hodgkin's lymphoma is particularly common in HCV infected patients[25,26], and over the last years the association between these two diseases has been confirmed in some meta-analyses[27,28]. The association between HCV infection and lymphoma seems to be greater in Mediterranean countries, Japan, and Brazil[29], while it seems less strong in the United Kingdom, Northern Europe, and Canada[30]. In the United States, two extensive studies showed a modest increased risk of developing B-cell non-Hodgkin's lymphoma in infected HCV patients compared to negative controls[31,32].

Other pathological conditions that have been associated with chronic HCV infection include autoimmune disorders (such as arthritis, Sjogren's syndrome, hemolytic anemia, autoimmune thrombocytopenia)[33], chronic kidney failure secondary to the onset of glomerulonephritis[24], cardiovascular diseases[34], endocrine disorders (such as thyroiditis), and type 2 diabetes mellitus[35].

THERAPY FROM INTERFERON MONOTHERAPY TO INTERFERON-FREE REGIMENS: BEGINNING OF A NEW ERA

The discovery of second-generation direct-acting antiviral agents (DAAs) was a real revolution in the treatment of hepatitis C (Figure 1). In the early 1990s, the use of alpha interferon (IFN) (3 million units three times a week for 6 mo) gave rather unsatisfactory results, with a sustained virological response rate (SVR) of about 25%[36]. The addition of ribavirin (RBV) combined with IFN 2α for 48 wk provided a good increase in the SVR rate, showing an efficacy of 43% compared to 19% of SVR in patients treated with IFN in monotherapy for 48 wk. However, patients receiving combination therapy showed a higher rate of adverse events (19% *vs* 13%) than patients in monotherapy, particularly anemia and leukopenia[37]. In the early 2000s, the use of pegylated IFN (peg-IFN) with the addition of RBV further improved viral eradication rates, with an SVR greater than 50%[38,39].

In 2011 the addition of protease inhibitors, such as boceprevir and telaprevir, to the standard therapy with peg-IFN + RBV allowed the achievement of eradication rates higher than 60% in the treatment of genotype 1 chronic HCV infection[40-43]. This triple therapy was, however, burdened by a greater number of adverse events than the standard therapy, particularly anemia, dysgeusia, and skin rash for boceprevir[41,44]. The use of telaprevir was more associated with the onset of skin rash, itching, anemia, nausea, and diarrhea[40,42]. In 2013 a new protease inhibitor, simeprevir, was used in combination with peg-IFN and RBV, which allowed a further increase in the success rate of antiviral therapy (86%) and a reduction of adverse events associated with previous regimens[45,46].

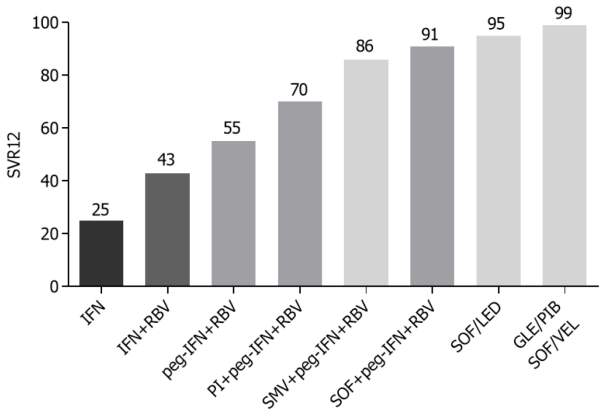


Figure 1 Sustained virological response rate of hepatitis C virus antiviral treatment, from interferon to direct-acting antiviral agents' regimens. SVR12: Sustained virologic response 12 wk post-treatment; IFN: Interferon; RBV: Ribavirin; peg-IFN: Pegylated interferon; PI: Protease inhibitor; SMV: Simeprevir; SOF: Sofosbuvir; LED: Ledipasvir; GLE: Glecaprevir; PIB: Pibrentasvir; VEL: Velpatasvir.

The big breakthrough came with the commercial introduction of sofosbuvir, a polymerase nucleotide inhibitor. Initially, it was used in combination with peg-IFN and RBV, obtaining a SVR rate of 90% and allowing a reduction of the treatment period to 12 wk[47-49]. It subsequently became the fundamental drug for the first IFN-free regimens[50], exceeding 95% of SVR in combination with ledipasvir in the treatment of genotype 1 HCV chronic infection[51]. Finally, a further step in the treatment of chronic HCV infection was the advent of pangenotypic regimens, such as sofosbuvir/velpatasvir and glecaprevir/pibrentasvir that guarantee an SVR rate close to 100% in all HCV genotypes[52-55]. The glecaprevir/pibrentasvir combination allowed a further reduction in the treatment period by showing a 99% of SVR in non-cirrhotic patients treated for 8 wk[56,57] (Table 1).

DAAS IN CIRRHOTIC PATIENTS

The second generation DAAs, thanks to their effectiveness and high safety profile, have allowed the treatment of patients once considered difficult to treat. In patients with compensated cirrhosis DAAs showed a 95% eradication rate, slightly lower (86.1% and 88.3%) in patients with Child Pugh score B[58,59]. Even in cirrhotic patients the treatment period has been further reduced. In a recent trial, the administration of glecaprevir/pibrentasvir for 8 wk in treatment-naïve patients with compensated cirrhosis showed efficacy and safety comparable to the 12 wk regimen [60]. A recent study of 667 patients with advanced/decompensated cirrhosis treated with DAAs showed an improvement of at least 3 points in the MELD score in 29% of patients at a long-term follow-up. This improvement was more frequent in patients with MELD score ≥ 16 at baseline (45%)[61]. In addition, DAA treatments reduced the hospitalization rate of patients with HCV-related liver cirrhosis[62] and allowed a reduction of liver transplantation for related HCV diseases in Europe from 22.8% in the IFN/RBV era to 10.6% in the first half of 2017[63]. Therefore, treating patients with compensated or decompensated cirrhosis with DAAs not only allows viral eradication, but also an improvement in the natural history of this disease.

IFN-free therapies have shown efficacy and safety even in patients with HCC. In a recent meta-analysis by He *et al*[64], 3126 patients with a prior history of HCC or with present HCC were compared with 49138 cirrhotic patients without HCC showing a percentage of SVR of 88.2% and 92.4%, respectively ($P > 0.001$). After initial controversy over the possibility that the DAAs could increase the occurrence or recurrence of HCC[65], in recent years several studies have denied this possibility. In a large cohort study of about 60000 patients, Ioannou *et al*[66] compared the incidence of HCC in patients treated with IFN-only, DAA + IFN, and DAA-only. Authors showed that SVR was associated with a significantly decreased risk of HCC in multivariable models irrespective of whether the antiviral treatment was DAA-only, DAA + IFN, or IFN-only. Particularly, DAA-induced SVR is associated with a 71% reduction in HCC risk.

Table 1 Direct-acting antiviral agents currently available in Italy and clinical indications (from Italian Association for the Study of the Liver[253])

DAAs	Clinical indications
Glecaprevir/Pibrentasvir	Pangenotypic regimen
	8 wk treatment in naïve/experienced (except GT3) non-cirrhotic pts and naïve CPT A5 cirrhotic pts
	12 wk treatment in experienced (except GT3) CPT A5 cirrhotic pts
	16 wk treatment in experienced GT3 non-cirrhotic or CPT A5 cirrhotic pts
	ESRD and/or hemodialysis (same treatment duration of pts without CKD)
	Contraindicated in decompensated cirrhosis
Grazoprevir/Elbasvir	GT1b, GT4
	12 wk treatment in GT1b or GT4 with HCV-RNA < 800000 IU/mL, naïve/experienced, CPT A5 cirrhotic or non-cirrhotic pts
	ESRD and/or hemodialysis (same treatment duration of pts without CKD)
	Contraindicated in decompensated cirrhosis
Sofosbuvir/Velpatasvir	Pangenotypic regimen
	12 wk treatment in naïve/experienced, cirrhotic or non-cirrhotic pts
	Indicated in decompensated cirrhosis
	Add RBV (if tolerated) in GT3 cirrhotic pts or in decompensated cirrhosis
	Optimal profile for drug interactions
	Contraindicated in pts with eGFR < 30 mL/min
Sofosbuvir/Velpatasvir/Voxilaprevir[1]	Pangenotypic regimen
	8 wk treatment in naïve/experienced non cirrhotic pts or GT3 naïve/experienced CPT A5 cirrhotic pts
	12 wk treatment in GT1, 2, 4, 5, 6 naïve/experienced CPT A5 cirrhotic pts
	First line therapy for retreatment after DAAs failure
	Contraindicated in pts with eGFR < 30 mL/min
	Contraindicated in decompensated cirrhosis

[1]In Italy, this regimen is currently refundable only for retreatment after direct-acting antiviral agent failure. DAAs: Direct-acting antiviral agents; GT: Genotype; pts: Patients; CPT: Child Pugh Turcotte Score; ESRD: End stage renal disease; CKD: Chronic kidney disease; RBV: Ribavirin; eGFR: Estimated glomerular filtration rate; HCV: Hepatitis C virus.

In a study involving 18076 patients treated with DAAs, with a follow-up of about 3 years, Kanwal *et al*[67] showed a cumulative incidence of HCC occurrence at 1, 2, and 3 years of 1.1%, 1.9%, and 2.8%, respectively. In patients with cirrhosis the cumulative incidence was 2.2%, 3.8%, and 5.6% at 1, 2, and 3 years, respectively. Two recent prospective Italian studies showed an incidence of HCC in patients treated with DAAs less than 3%, and in both studies the lack of SVR was a risk factor for the development of HCC[68,69].

Careful screening for hepatic nodules is essential before starting treatment with DAAs as the presence of undefined/benign hepatic nodules is significantly associated with HCC occurrence during or after treatment with DAA[70,71]. The recurrence rate of HCC in patients treated with DAAs is quite varied in literature, ranging from 2.2% in a cohort of liver transplant recipients for HCC and then treated with DAAs to 45.1% in cirrhotic patients[72]. In a broad, retrospective cohort study, patients treated with DAAs were compared with HCV-infected, cirrhotic patients that had not received any antiviral treatment, showing a lower early recurrence rate of HCC in the group of patients treated with DAAs (42.1% *vs* 58.9%)[73]. Finally, treatment with DAAs showed an improvement in survival in cirrhotic patients with HCC compared to patients with HCC and chronic HCV infection not treated with DAAs, thus resulting in effective, safe and beneficial treatment for patients[74-76].

IFN-FREE REGIMEN IN EXTRAHEPATIC MANIFESTATIONS

DAA regimen has been shown to be highly effective in treating patients with chronic HCV infection and the concomitant presence of extrahepatic manifestations, such as mixed cryoglobulinemia and/or lymphoma. The SVR rate is similar compared to patients with no extrahepatic symptoms, exceeding 90%[77]. In a study by Passerini *et al*[78], 93 patients with chronic HCV infection and mixed cryoglobulinemia treated with DAAs were enrolled and compared with a control group of 89 HCV infected without mixed cryoglobulinemia also treated with an IFN-free regimen. The SVR rate was superimposable between the two groups, and the percentage of adverse events was similar. The authors demonstrated the high efficacy and safety of DAA treatment in patients with mixed cryoglobulinemia.

Conflicting data have been collected on the effectiveness of DAA regimen in the treatment of mixed cryoglobulinemia. In a study involving 148 patients with HCV infection and secondary mixed cryoglobulinemia vasculitis treated with DAAs with an average follow-up of about 15 mo, it was observed that 72.6% of patients obtained a complete response (defined as improvement of all organs involved at baseline without subsequent relapse), 22% a partial response, while 5% had no clinical response. Cryoglobulins were eliminated in the blood in about 50% of patients, while in over 90% of patients vasculitic and renal manifestations were eliminated; in 86% of patients an improvement of arthralgia was observed. Factors associated with a partial or absent response were a severe form of vasculitis and peripheral neuropathy[79]. In a recent work by Colantuono *et al*[80], 70 patients with HCV and mixed cryoglobulinemia vasculitis treated with DAAs were compared with 39 patients with the same clinical condition treated with IFN. The percentage of patients achieving at any time point of follow-up (more than 2 years) a complete or partial response was similar in the two groups, but patients treated with an IFN-free regimen had less effectively eliminated cryoglobulins and B-cell clones in the blood. Most importantly, they had a significantly higher percentage of vasculitis relapse (18% *vs* 3%, $P = 0.028$) and a greater need for treatment with rituximab.

In 2017, our group demonstrated that DAA therapy was safe and effective in patients with chronic HCV infection and with diffuse large B cell non-Hodgkin lymphoma (DLBCL) in current chemotherapy. In this small cohort of patients the SVR rate was 100%, and it emerged that patients treated with DAAs had a greater disease-free survival than HCV+ patients with DLBCL not treated with antiviral therapy (historical cohort). In multivariate analysis, DAAs have proven effective ($P = 0.012$) in improving remission of aggressive lymphoma in HCV+ patients[81]. Our data were subsequently confirmed in a study by Pellicelli *et al*[82], in which they demonstrated how HCV+ patients with DLBCL treated with DAAs or with peg-IFN + RBV who had not obtained SVR showed a greater relapse of DLBCL after chemotherapy than patients who reached a SVR. Furthermore, an Italian-French cohort of 47 HCV+ patients with DLBCL showed an SVR rate of 96% and a progression of lymphoproliferative disease in only three patients who had obtained SVR[83].

In a recent paper by Frigeni *et al*[84], 100 HCV+ patients with indolent non-Hodgkin lymphoma treated with an IFN-free regimen were enrolled. They showed an SVR rate of 99%, with an overall hematological response rate of 66%, with 23% of complete response and 43% of partial response; a higher overall hematological response rate was observed in marginal zone lymphoma than in non-marginal zone lymphoma (73% *vs* 48%, $P = 0.021$). Moreover, in this study a comparison between patients treated with DAAs alone as first-line therapy for indolent non-Hodgkin lymphoma (excluding patients previously treated with IFN) and a historical cohort of HCV+ patients with indolent non-Hodgkin lymphoma treated with IFN was performed. Although the SVR rate was higher in patients treated with DAAs, IFN therapy had a higher percentage of complete response and a higher median duration of response than DAAs, thus demonstrating a greater anti-lymphoma action than IFN-free regimens, secondary to the well-known antiproliferative activity of IFN. The hematological outcomes in terms of overall survival and progression-free survival (PFS) were overlapping[84]. Finally, a recent meta-analysis of our group comprising 317 HCV+ patients with non-Hodgkin lymphoma treated with DAAs and/or an IFN-based regimen and 250 controls not treated with antiviral therapy showed how the achievement of an SVR, regardless of the antiviral treatment performed, has a strong positive effect on the non-Hodgkin lymphoma outcome in terms of progression-free survival[85].

DAAS IN PATIENTS WITH CHRONIC KIDNEY DISEASE

DAAs are effective and safe in patients with chronic kidney disease (CKD) and/or hemodialysis. Antiviral treatments containing sofosbuvir are currently not recommended as first-line therapy in patients with CKD and or end stage renal disease due to its renal metabolism and possible adverse events on kidney function[86]. The first combination to be used was ombitasvir/paritaprevir/ritonavir + dasabuvir in the treatment of patients with genotype 1 and 4[87]. Subsequently, the combination grazoprevir/elbasvir showed efficacy and safety in patients with CKD and HCV chronic infection genotypes 1 and 4[88,89]. The pangenotypic regimen glecaprevir/pibrentasvir showed high SVR rates and satisfactory safety profile in the treatment of moderate/advanced CKD and/or hemodialysis patients with HCV chronic infection genotype 1-6 for 12 wk of treatment[90]. In a recent trial, the administration of glecaprevir/pibrentasvir for 8 wk in non-cirrhotic patients with moderate/advanced CKD and/or in hemodialysis with chronic HCV infection (all genotypes) showed SVR rates comparable to the 12 wk, thus allowing a significant reduction in the treatment period[91]. The effectiveness and safety of glecaprevir/pibrentasvir in patients with CKD or end stage renal disease has also been tested in real life settings[92,93], showing data superimposable with controlled trials. The efficacy and safety of sofosbuvir/velpatasvir has recently been tested in patients with CKD and/or hemodialysis. Although the two cohorts are limited (51 and 59 patients, respectively), the SVR rate was high (96% and 95%) with an acceptable safety profile[94,95]. Studies with larger populations will be needed to confirm these data.

NEW TARGET: ELIMINATION OF HCV INFECTION. A PATH INTERRUPTED BY THE GLOBAL PANDEMIC

In 2016, the WHO set the challenge of eliminating HCV infection and blocking its spread worldwide by 2030[96]. In order to achieve this objective, the European Association for the Study of the Liver has proposed to strengthen the measures of case finding and treatment in populations at greater risk of infection, such as people who inject drugs, prisoners, the homeless, and men who have sex with other men, so as to obtain a microelimination in these populations and subsequently the macroelimination of HCV infection[97]. In a recent study of our group in which 160 people who inject drugs with HCV infection, afferent to drug addiction centers were treated, the SVR rate was 98.5% without significant side effects[98]. Furthermore, the concomitant opioid substitution therapy does not affect the efficacy and safety of DAAs[99]. Even in a complicated context such as prison, therapy with DAAs has proved highly effective, with a 100% SVR in 46 convicts in southern Italy. This study highlights how difficult it is to reach these patients not to treat them[100].

The conditions for achieving microelimination in 2030 were quite satisfactory, but the recent global pandemic caused by severe acute respiratory syndrome coronavirus 2 infection has suddenly interrupted this process. Indeed, the national health systems have had to concentrate all their resources (human, structural, and economic) on the fight against this infection, temporarily putting aside the activities of screening and treatment of chronic diseases, such as chronic HCV infection. In a study conducted at the Boston Medical Center, it was shown that between March and June 2019, there was a 49% reduction in the performance of HCV tests and a 42% reduction in new HCV+ patient identification hospital wide. These numbers are even higher in ambulatory clinics connected to this center for screening programs for HCV infection with a reduction of 72% and 63% in the number of HCV tests performed and in the new HCV+ patient identification, respectively[101].

In Italy, a recent survey proposed by the Italian Association for the Study of the Liver showed that outpatient activities concerning viral hepatitis were reduced in 99% of hospitals involved in the study, and the start of antiviral treatment has been postponed in 23% of centers, even for patients considered at risk of disease evolution [102]. In Germany between March and May 2020 there was also a sharp reduction in outpatient hepatological activities and in the prescription of anti-HCV treatments [103]. The consequences of this reduction in the screening and treatment of hepatitis C could be serious in the near future. In a study by Blach *et al*[104], it was estimated that in the decade 2020-2030 there could be 900000 missed new diagnoses of HCV infection and a reduction of about 750000 treatments due to the global pandemic. This could result in an increase in end-stage disease outcomes, with 44800 excess HCC cases and 72300 excess liver-related deaths predicted over 2020-2030.

HEPATITIS B

HBV, the pathogen responsible for hepatitis B, is a major public health problem, as both its acute and chronic infections are still responsible for numerous cases of morbidity and mortality. International guidelines provide specific indications (with few differences from one another) on antiviral therapy against HBV, but this field still represents an open challenge because despite the increasing effectiveness of anti-HBV drugs approved over the years, a definitive cure for hepatitis B does not yet exist.

EPIDEMIOLOGY

In 2016, a worldwide prevalence of hepatitis B surface antigen (HBsAg) positivity of about 292 million people was estimated and that in the same year only 10% of these were identified[105]. Moreover, it was calculated that this virus caused 887000 deaths in 2015, mainly from cirrhosis or HCC. Africa and the Western Pacific are the areas with the higher prevalence of infection, and the WHO African Region was estimated to have the lowest vaccine birth dose coverage[105,106]. Despite the open challenges, important results have already been achieved, such as the reduction in chronic hepatitis B (CHB)[106-108], HCC[109], extrahepatic manifestations, and decreased mortality for fulminant hepatitis[110] due to vaccination against this virus.

HBV can spread in different ways. In high prevalence areas, transmission during the first years of life is the most common. In this age group, they can be distinguished by perinatal transmission (from infected mother to newborn), that is the most widespread worldwide and occurs at the time of birth, shortly after, or rarely in the intrauterine life and horizontal transmission during childhood that is prevalent in certain countries and can occur *via* small lesions through contaminated environmental surfaces[106,108]. Horizontal infections in adulthood occur mainly through the parenteral route in both health care settings (due to the use of contaminated blood derivatives or contaminated instruments) or in the context of intravenous drug use, such as opioids. Getting tattooed and performing piercings are other procedures that can allow HBV spread [106]; sexual intercourse is a consistent risk factor for certain categories of people, such as male homosexuals or people who have multiple partners[106,108]. In addition to blood, HBV is found in saliva, seminal, vaginal, or menstrual fluids[106,111].

HISTORY, CLASSIFICATION, AND VIROLOGY

In 1965, Blumberg *et al*[112] described the formation of precipitins (precipitating antibodies) after contact between serum from two hemophilic patients and that of an Australian aborigine. Since this antigen (called "Australia antigen") was subsequently found also in the serum of patients suffering from acute hepatitis, it was hypothesized that it could be linked to a hepatitis virus[113]. Following observations through electron microscopy, in 1970 David Dane made it known for the first time that the agent responsible for the "Australian antigen-associated hepatitis" was a virus of 42 nm in diameter[114], therefore called "Dane particles." The term "hepatitis B virus," however, appeared several years before identification of this virus (in 1947) and was proposed to distinguish the two different types of hepatitis that were known at the time[115,116].

HBV belongs to a family of hepatotropic, DNA viruses named Hepadnaviridae. This family includes five genera: Avihepadnavirus, Herpetohepadnavirus, Metahepadnavirus, Orthohepadnavirus, and Parahepadnavirus. The genus Orthohepadnavirus comprises 12 species (which can be hosted by a wide range of mammals) among which there is the HBV species that infects humans and apes[117,118]. HBV is divided into four serotypes (adw, ayw, ayr, and adr) based on HBsAg and depending on nucleotide sequences in ten different genotypes (A, B, C, D, E, F, G, H, I, J)[118,119]. There are some subtypes of the different genotypes. The various genotypes have a different geographical distribution, are associated with different responses to therapies (genotypes A and B have a better response to IFN than C and D), and outcomes (genotype C is associated with an increased risk of advanced fibrosis and HCC)[119].

The HBV genome consists of a 3.2 kb long, partial double stranded, relaxed circular DNA molecule, formed by a complete coding negative strand, linked to the viral polymerase, and an incomplete noncoding positive strand. The minus strand contains four open reading frames (ORF) that partly overlap: preC/C, P, preS/S, and X[117,119,120].

PreC region (pre-core) encodes the E antigen (HBeAg) which unlike HBc (that remains in the cell) is secreted into the circulation; this protein has immunomodulatory/tolerogenic functions, and it is believed to give the virus an evolutionary advantage (in fact it is found in all Orthohepadnaviruses). It is still unclear if it could also have other roles[121,122]. ORF C encodes the highly antigenic and self-assembling core protein HBc, which constitutes the structural protein (HBcAg) that covers the HBV DNA forming the nucleocapsid. It also participates in other steps of the HBV life cycle, including viral replication[123]. ORF P encodes the viral polymerase (P protein), a multifunctional protein responsible for viral replication; this latter is divided into four (TP, spacer region, RT, and RNase H) domains that allow the DNA or RNA reverse transcription and the ribonuclease activity[124]. Pre-S (1 and 2)/S ORF encode the HBsAg that form the viral envelope; they are distinguished in small, medium, and large surface proteins. Small surface proteins are the most abundant envelope protein and includes the "a" determinant, which is the main target of the neutralizing anti-HBs response. HBV results in an icosahedral shaped, enveloped particle of about 42 nm in diameter[125]. ORF X encodes the HBx protein, whose functions have long been unclear; it can transactivate several viral and cellular gene promoters and cause epigenetic modifications of covalently closed circular DNA (cccDNA)[126].

HBV LIFE CYCLE

The life cycle of HBV has been described in detail. Briefly, the virus initially attaches to the hepatocyte surface through a low affinity binding of S-HBsAg with heparan sulfate proteoglycans. Glypican-5 was recently found to be involved in the early stages of HBV attack on the host cell. Hence, a highly specific bond between L-HBsAg and sodium-taurocholate cotransporting polypeptide takes part, and this is followed by entry *via* endocytosis[120,127,128]. Once inside the cell, the fusion of the envelope with the endosome membrane causes the release of the nucleocapsid into the cytoplasm, and subsequently the HBV relaxed circular DNA is transported to the nucleus. After completion of the positive strand, the HBV DNA is converted into a cccDNA molecule that remains stable in the nucleus in the form of a chromatin-like minichromosome [120,129]. Here it can undergo epigenetic modifications that modify its expression. The cccDNA probably persists for the entire life of the hepatocyte and explains the persistence of this virus and the viral reactivations that occur in certain patients, such as immunosuppressed or those who stop antiviral therapy[128]. The cccDNA serves as a template for the transcription of viral mRNAs by the host's RNA polymerase II. The envelope and the HBx proteins originate from the subgenomic RNAs, while the core protein and viral polymerase derive from pregenomic RNA (pgRNA). This latter is also encapsulated together with HBV polymerase by core proteins to form immature particles. After encapsulation, the pgRNA will serve as a template for the reverse transcription of HBV DNA that is first synthesized in its minus strand[120,128,129]. The latter acts as a template for the synthesis of the positive HBV strand, which will result as partially incomplete. These nucleocapsids are supplied with envelope proteins in the endoplasmic reticulum (ER), and further modifications occur in the Golgi complex[120]. Finally, complete particles and sub-viral particles (including "S" antigen) are released from the host cell. Other nucleocapsids, on the other hand, recirculate in the nucleus to supply it with viral genome. HBV is not cytopathic, and liver damage is mediated by the immune system[107].

CONVENTIONAL AND NEW MARKERS

In the acute HBV infection, the appearance of HBV DNA is followed by HBsAg and HBeAg (the latter, as well as HBV DNA, is a marker of viral replication). HBsAg appears and increases approximately by 1-10 wk after contact with HBV, and anti-HBc IgM and then IgG are found in the serum about 2 wk after the appearance of HBsAg [130,131]. HBV DNA and HBsAg persist for the time of duration of clinical manifestations, while HBe seroconversion usually coincides with the peak of clinical illness [131]. Following the disappearance of HBsAg, anti-HBc IgM may be the only detectable marker. In those who develop them, anti-HBs appear during or after recovery from the disease; they are a marker of immunity against HBV and are found together with anti-HBc IgG in those who had a past infection or alone in vaccinated people[131].

An occult HBV infection (OBI) is defined by the combination of HBsAg absent/HBV DNA present in the liver (*e.g.*, cccDNA) and HBV DNA absent or present in the serum. This phase is distinguished in seropositive OBI [(anti-HBc and/or anti-HBs positive) that may occur both after overcoming an acute infection or following a chronic infection/hepatitis] and seronegative OBI [(anti-HBc and anti-HBs negative) that may result from the progressive loss of antibodies against HBV or because they never developed][132]. It is of crucial importance to recognize an OBI because of the high risk of viral reactivation that exists for such patients in certain circumstances (*e.g.*, anti-CD20 chemotherapy regimens for hematological malignancies)[133].

HBsAg persistence for at least 6 mo is defined as chronic hepatitis B[108,131]. In this case, the HBeAg and HBV DNA persist for a long time. Levels of HBsAg vary in the different phases of the disease and as well as the values of HBV DNA correlate with the risk of developing cirrhosis and HCC[134,135]. As discussed below, HBsAg loss is associated with a reduction (but not abolition) in the occurrence of HBV related complications, but it is a rare event. Furthermore, it is not yet known whether the appearance of anti-HBs confer greater advantages in terms of clinical outcomes than the disappearance of HBsAg alone[135]. In recent years, new biomarkers have been studied to overcome the limitations offered by those currently in use.

HBcrAg is made up of three related viral proteins (HBcAg, HBeAg, and p22cr; the latter is a 22-kDa pre-core protein) that share a 149 amino acids long identical sequence. HBcrAg measurement is not redundant to that of HBsAg, and this marker can also be detected in HBsAg negative patients. It showed an association with intrahepatic cccDNA, intrahepatic pgRNA, HBV translational activity, and serum HBV DNA[136]. From a practical point of view, higher levels of HBcrAg were found to be associated with an increased risk of HCC onset [in nucleos(t)ide analogues (NAs) treated or untreated patients] and recurrence. Moreover, this marker was linked to the risk of viral reactivation, response to treatment, and relapse after therapy withdrawal [136-138]. The latest guidelines for the management of HBV infection of the Japan Society of Hepatology recommend HBcrAg dosage to predict the risk of relapse after cessation of therapy, while in Europe and the United States it has not yet entered into daily practice. Other tools that have been explored in the context of HBV infection include circulating HBV RNA (prediction of response to treatment and relapse after the end of treatment), anti-HBc titers, and HBeAg levels[138].

CLINICAL MANIFESTATIONS OF HBV INFECTION

This virus causes acute and chronic infections. After contact with HBV, the incubation period is approximately 75 d, but it can be very variable[106]. Acute hepatitis B can be asymptomatic or symptomatic, with varying severity. Newborns and children usually have an asymptomatic and anicteric course, while adults can develop a symptomatic hepatitis in 30%-50% of cases. Fulminant hepatitis occurs in about 0.1%-0.5% of infected patients[131,139]; it is probably due to an abnormal response of the immune system and has a very high rate of mortality. In symptomatic patients, the disease can run through the typical course of acute hepatitis (incubation, prodromal, icteric, and resolution periods). A few weeks after the appearance of viral markers in the serum, abnormalities of liver biochemistry can be found[131]; transaminases can exceed 1000 IU/L [with alanine aminotransferase (ALT) greater than aspartate aminotransferase], bilirubin increase can be of varying degrees depending on the severity of the disease, whilst an increase in international normalized ratio (> 1.5) is typical of severe forms [107]. Clinical and laboratory resolution occur in highly variable timescales, depending on the severity of the disease. In adults, the disease spontaneously resolves itself in more than 95% of cases, and this is followed by the appearance of anti-HBs.

Progression to chronic hepatitis mainly depends on the age at which the infection is acquired. It occurs in about 90% of cases if the infection is acquired in the perinatal or infantile period, 30%-50% if acquired by children up to 6-years-old, and less than 5% if acquired in adulthood[106,139]. Chronic hepatitis B proceeds through various phases characterized by different laboratory findings, and chronically HBV infected and untreated people have a 5-year cumulative incidence of cirrhosis of 8%-20%. Therefore, the disease can further progress to the onset of complications and HCC. The latter was found to arise with an incidence rate of 2%-5% per year in HBV cirrhotic patients[106,107]. Viral, host, or environmental related factors (genotype C, older age, male gender, coinfection with HDV, HCV, and HIV, alcohol, diabetes, and others) can increase the probability of worse outcomes, including HCC[107,139]. Several extrahepatic manifestations can be observed in the context of HBV infection and are

thought to be due to the formation of immune complexes with HBsAg or HBeAg. Among these, there are systemic syndromes (serum sickness-like or flu-like), vasculitis (*e.g.*, polyarteritis nodosa), glomerulonephritis, skin manifestations (*e.g.*, purpura and lichen planus), polyarthritis, neurological disorders (*e.g.*, Guillain-Barré syndrome), cryoglobulinemia, and increased inflammatory indices[107,140]. It has also been found that HBV infected people have a higher risk of developing B-cell lymphoma[141].

PHASES OF CHRONIC INFECTION

HBV chronic infection is a dynamic condition, and its course depends on the virus-host relationship. To characterize the disease and choose whether to start the treatment, viral (HBsAg, HBeAg, anti-HBe, HBV DNA) and liver damage (ALT, fibrosis, or necroinflammation through non-invasive tests such as liver elastography or score results or with liver biopsy) markers must be evaluated. Based on the results of these tests, various phases (Table 2) of CHB can be identified[107,142].

The last phase, described above, is the occult phase (also named HBsAg loss phase, resolved CHB, or phase 5)[109,143,144]. Gish *et al*[144] also proposed two theoretical phases: "clearance of cccDNA" and "clearance of cells that have integrated HBV." Progression from one phase to another is not necessarily sequential (*e.g.*, the phase 2 can be followed by phase 3 or directly by phase 4), and the return to a previous phase is also possible. Moreover, in daily practice, several cases of CHB may not belong to any of these phases (for example during transition between phases or in the presence of concomitant liver diseases)[107,143].

HBV AND HCC

HCC has variable incidence rates worldwide, depending on the prevalence of the various risk factors around different geographical areas. Most cases, however, are documented in the Asian regions[145]. In 2015, there were about 854000 incident cases of HCC, and this cancer caused 810000 deaths worldwide; these numbers are expected to increase in the coming years[146]. In most cases, HCC develops in the context of cirrhosis, but it is well known that many HCCs arise in the absence of such disease. Although the prevailing causes of HCC vary by region, HBV is the overall leading cause. Despite an observed increase in the overall number of cases in the 1990–2015 time frame, a deflection of incidence rates of liver cancer due to HBV was also reported during the same period[146]. This virus can cause HCC in the absence of cirrhosis and at a younger age compared to other etiologies[147]. In addition to viral, host, and environmental risk factors mentioned above, several conditions have been associated with the onset of HBV-related HCC[148]: host genetic predisposition (*e.g.*, single nucleotide polymorphisms). Immune response to HBV, cell death, hepatic inflammation, and liver fibrosis create an optimal substrate for the onset of HCC. Mutations in the HBV preS1/S2 domain cause an accumulation of altered large surface proteins in the ER, resulting in the development of oxidative stress. Integration of HBV-DNA into the host genome causes genomic instability or direct involvement (insertional mutagenesis) of cancer-associated genes (*e.g.*, *TERT*, *MLL4*, *CCNE1*).

Viral proteins (HBx, preS and HBc) interfere with critical steps of cell life cycle.

The HBx protein can have a procarcinogenic role as it causes reactive oxygen species production in mitochondria and ER stress. It can interfere in cancer-related pathways (*e.g.*, Jak/STAT, RAS/RAF/MAPK, or Wnt/β-catenin; moreover HBx is able to inactivate p53) and can cause epigenetic modifications of the host DNA. It alters the expression of microRNAs and long noncoding RNAs, and it interferes with processes involved in DNA repair. It was proposed to promote the "stemness" of HCC cells[147-149]. Much knowledge on HBV-related HCC has been accumulated, but there is still a gap compared to what is clinically useful.

CURRENTLY AVAILABLE THERAPIES, GOALS, AND UNRESOLVED ISSUES

The aim of antiviral therapies against HBV is to increase the survival of people suffering from CHB by reducing cases of liver failure, cirrhosis, and HCC and prevent the transmission of the infection. Specific terms are used to indicate the goals of

Table 2 New classification of chronic hepatitis B phases

HBsAg+ ≥ 6 mo	
HBeAg-positive chronic infection (1)	
HBe: HBeAg+; HBV DNA: very high; ALT: persistently normal; Liver disease: minimal or absent	Ex "immune-tolerant;" Typical of children and young adults infected at birth; HBV DNA: > 1 million IU/mL; highly contagious people; Low rate of spontaneous HBeAg loss.
HBeAg-positive chronic hepatitis (2)	
HBe: HBeAg+; HBV DNA: high; ALT: elevated; Liver disease: moderate or severe	Ex "immune reactive HBeAg-positive;" People infected in adulthood are likely to enter this phase; HBV DNA > 20000 IU/mL; rapid progression to fibrosis; Frequent HBe seroconversion.
HBeAg-negative chronic infection (3)	
HBe: HBeAg-, anti-HBe+; HBV DNA: low or undetectable; ALT: normal; Liver disease: low	Ex "inactive HBV carrier;" HBV DNA: < 2000 IU/mL; it can also be > 2000 IU/mL but usually < 20000 IU/mL; Low risk of progression to cirrhosis or HCC; HBsAg loss in 1%–3%/yr.
HBeAg-negative chronic hepatitis (4)	
HBe: HBeAg-, anti-HBe+; HBV DNA: moderate or high; ALT: elevated (variable); Liver disease: moderate or severe	HBV DNA: 2000-20000 IU/mL; Older people, frequent mutant precore HBV; Fluctuating levels of HBV DNA and ALT; Rare spontaneous remission and rapid progression to cirrhosis.

HBV: Hepatitis B virus; HBeAg: Hepatitis B E antigen; HBsAg: Hepatitis B surface antigen; ALT: Alanine aminotransferase; HCC: Hepatocellular carcinoma.

antiviral therapy. With the currently available drugs a "definitive," "sterilizing," or "true" cure for hepatitis B (defined by HBsAg/serum HBV DNA/hepatic cccDNA/integrated HBV DNA not detected) is not possible, given the persistence of HBV DNA in the nucleus[107,150]. The "functional cure" corresponds to HBsAg loss, with or without seroconversion, and undetectable HBV DNA following a course of antiviral therapy. This event, related to both therapy and disease factors, is associated with reduced risk of HCC, liver failure, and other liver-related adverse outcomes[151]. This "ideal endpoint" is uncommon with currently available drugs, but after HBsAg disappearance (both after therapy with NAs or peg-IFN or spontaneous) this result is generally stable and allows patients to stop the therapy[107,135,143]. Experts believe that near-future therapies will have to achieve this goal rather than others (that however may appear more radical), and it is believed that HBsAg loss should be the primary endpoint of phase III trials[150]. In a recent meta-analysis that included 42588 CHB adult patients, it was observed that HBsAg loss occurred in 3194 of them, with an annual pooled seroclearance rate of 1.02%. This event was more likely in patients who were HBeAg negative and those who had lower HBsAg and HBV DNA levels at baseline. On the other hand, the treatment did not have a major effect in achieving this goal[151]. Other studies also obtained similar results regarding the rate of spontaneous or post therapy HBsAg loss. In any case, even after HBsAg loss, the risk of HCC is not eliminated[107,143].

Therefore, the main and "realistic" endpoint that current therapies can offer correspond to suppression of HBV DNA or virological response, defined in different ways depending on type and timing of therapy. Normalization of ALT (biochemical response), loss of HBeAg ± seroconversion, or reduction in necroinflammatory activity (histological response) are other surrogate but important endpoints. The indications for treatment (Figure 2) derive mainly from ALT, HBV DNA values, and from the severity of liver damage defined by liver biopsy or non-invasive tests such as transient elastography or scores such as FIB-4 and FibroTest[107,142]. A recent meta-analysis that included 162 studies and 145789 people with chronic HBV infection from all WHO regions estimated that 19% of infected people were eligible for treatment according to current indications[152].

Antiviral therapy is usually not indicated in those who have HBeAg-positive (ex-immunotolerant phase, IT) or negative (ex-inactive carrier phase) chronic infection [107,150]. IT patients who remain in this phase showed a minimal or no progression of liver disease[153]. Some studies seemed to provide evidence against such indications, showing, for example, a greater risk of worse outcomes in untreated IT patients than those in immune-active phase on NAs[154]. However, these and conflicting results from other studies were partly attributed to patient inclusion criteria[155]. Anyhow, it is not excluded that despite current statements IT patients may benefit in the future from more effective therapies to reduce the viral replication (and the risk of HBV

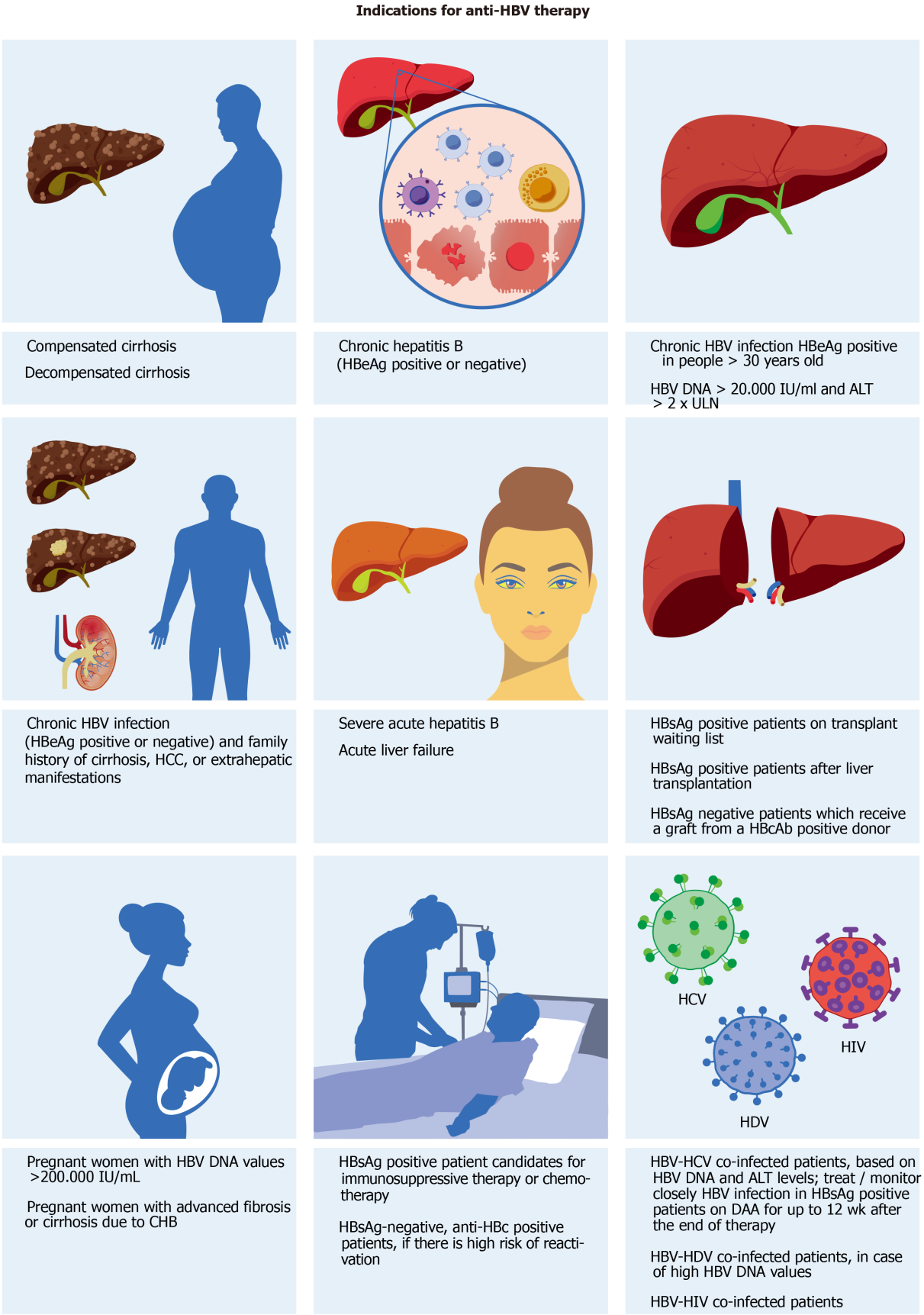


Figure 2 Indications for anti-hepatitis B virus therapy according to international guidelines. HBV: Hepatitis B virus; HCV: Hepatitis C virus; HDV: Hepatitis D virus; HCC: Hepatocellular carcinoma; HBeAg: Hepatitis B E antigen; HBsAg: Hepatitis B surface antigen; CHB: Chronic hepatitis B; ALT: Alanine aminotransferase; ULN: Upper limits of normal; DAA: Direct-acting antiviral agents.

transmission) and potential complications[150].

The drugs currently used for the treatment of HBV infection are: NAs: nucleoside analogues [lamivudine, telbivudine, entecavir (ETV)]; nucleotide analogues [adefovir dipivoxil, tenofovir disoproxil fumarate (TDF), tenofovir alafenamide fumarate; besifovir dipivoxil maleate was recently approved in Korea]; and peg-IFNα.

Between these options, the most used are NAs[107,119]. This class of drugs inhibits reverse transcription activity of HBV DNA polymerase; NAs compete with natural substrates, and their integration in the nascent viral DNA chain interrupts its formation[156]. Lamivudine, adefovir dipivoxil (the first two approved NAs for the treatment of hepatitis B), and telbivudine have a low barrier to resistance and therefore are characterized by a high percentage of cases of resistance during therapy (70% for lamivudine and 29% for adefovir dipivoxil after 5 years of therapy)[107,157]. Despite this, these drugs are still used in some countries mainly for their low cost[157].

On the other hand, ETV, TDF, and tenofovir alafenamide fumarate, the most powerful NAs available today, have a high barrier to resistance. They are associated with higher HBV DNA suppression rates and are the recommended first-line drugs for treatment of CHB. These NAs have a high tolerability and can be used regardless of the severity of the liver disease (even in decompensated cirrhosis) and comorbidities [107]. The occurrence rate of resistance to ETV after 5 years of therapy was shown to be approximately 1% in nucleoside naïve CHB patients, while no cases of resistance were recorded in a 7 year-long study in which 437 CHB patients received TDF monotherapy[158] and in other long-standing studies[107]. Accordingly, the discovery of tenofovir resistant strains would be of considerable impact.

Two cases of viral breakdown addressed to a quadruple mutation (CYEI) were recently reported in CHB patients and linked to the ongoing therapy, which included TDF. It was therefore emphasized that in these cases there would be no consolidated possibilities of therapy[159]. In a response study, however, it was pointed out that CYEI mutation was already documented before the availability of TDF, suggesting that it could (rarely) arise in therapy naïve people. Other evidence behind the reduced susceptibility/resistance to tenofovir has recently been claimed, but currently the clinical impact of this phenomenon is limited[160,161].

Although NAs have been shown to effectively suppress HBV DNA and reduce the progression of liver disease, the disappearance of HBsAg, as already mentioned, rarely occurs, and they have no direct effect on cccDNA. Nevertheless, a study on patients on long-term therapy with NAs showed that they can achieve a reduction in intrahepatic HBV DNA and cccDNA and that the latter can also become not detectable[162]. It was recently observed, however, that among patients that were part of the same study who had undetectable cccDNA, all those who discontinued antiviral therapy (n = 13) experienced an HBV DNA rebound, unlike those who continued treatment. These results suggested that even small, not detectable amounts of cccDNA may allow the virus to reactivate[163]. NAs require frequent monitoring, and usually they must be assumed for an indefinite period. Moreover, the risk of HCC is not eliminated, and long-term therapy is associated with some adverse events. To reduce renal or bone toxicity, ETV or tenofovir alafenamide fumarate should be preferred over TDF[107,142].

Conversely, peg-IFNα is administered for a finite period, generally 48 wk, but longer durations are also recommended in certain cases[107]. The choice between peg-IFNα and the NAs depends on the patient and his preferences, previous treatments, virological factors, and the liver disease. However, it requires subcutaneous injection, and it is associated with well-known side effects. Moreover, due to its non-optimal safety profile, it is contraindicated in patients with decompensated cirrhosis, in cases of other co-morbidities (*e.g.*, autoimmune diseases, psychiatric disorders, cytopenias), during pregnancy, and in severe acute hepatitis B or liver failure, and it is not recommended in some patients with compensated cirrhosis (*e.g.*, those with portal hypertension)[107,142]. Combination therapy (NA-IFN) increased response rates but is currently not recommended as further confirmations are needed[107,119]. The response to anti-HBV therapy and its effect on liver-related outcomes, such as the onset of HCC, were found to vary also by ethnicity (*e.g.*, Asian patients are less likely to experience HBsAg loss following NA therapy than Caucasian ones)[107,119,150].

FUTURE PERSPECTIVES

Many molecules are being studied as a therapy for hepatitis B to improve the results obtainable with the drugs currently available. Among the various approaches, some aim to strengthen the immune response against HBV, while others interfere with

specific steps in its life cycle. Direct elimination of the HBV genome is not yet close to clinical practice[119,164].

HBV entry inhibitors: Bulevirtide binds and inhibits the sodium-taurocholate cotransporting polypeptide, preventing the intrahepatic entry of HBV. The same mechanism of action is shared by Cyclosporin A and its analogues, but the block of bile acids transport should be considered. Other strategies to block cell entry include attack inhibition and antibodies against HBV antigens.

cccDNA inhibitors: TALENs and CRISPR/Cas9 can lead to genome rearrangement by acting in specific sites and are under investigation to evaluate their efficiency in targeting cccDNA. In these cases, the potential risk of making changes to non-target genes has to be considered.

Core protein assembly/allosteric modulators: these molecules interfere with the formation of viral capsid. The class I core protein assembly/allosteric modulators lead to the appearance of abnormal, misassembled, capsid structures, while the class II core protein assembly/allosteric modulators accelerate the formation of morphologically intact but empty capsids (without pgRNA).

RNAi-based agents: in this case HBV transcripts are recognized and degraded by small RNA molecules, resulting in post-transcriptional silencing.

HBsAg release inhibitors: REP 2139 inhibits the release of HBsAg by targeting sub-viral particles, with the aim of counteracting its tolerogenic effect.

RNaseH inhibitors: they inhibit the RNase activity of DNA polymerase, an important step for viral replication. Other options under study include new NAs, innate response stimulating drugs (TLR-7, TLR8, or STING agonists, RIG-1 activator), and therapeutic vaccines.

HBV/HDV INFECTION

Hepatitis D, or delta, is the liver disease caused by HDV that was discovered by Rizzetto *et al*[165] in 1977. A recent review and meta-analysis estimated a 4.5% prevalence of anti-HDV among HBsAg-positive people, corresponding to about 12 million HDV infections worldwide. Other recent estimates, however, provided much larger numbers[166,167]. Its real spread remains elusive probably due to the non-systematic and universal screening in HBsAg positive patients[166,167]. Over recent years a drop in the prevalence and incidence of HDV infection in developed countries, such as Italy, was recorded as presumably the result of HBV vaccination and the increased sensitivity and precautionary measures to counteract risk factors for parenteral and sexually transmitted infections[167-169]. A current issue is delta hepatitis cases due to immigration from high prevalence countries. Nowadays, this infection is highly prevalent in several Asian countries (*e.g.*, Mongolia), West and central Africa, Moldova, and the Amazon region[167-169].

HDV is a defective, satellite virus, 36-43 nm in diameter, which belongs to the Deltaviridae family that in turn includes a single genus (Deltavirus). The latter contains a single species, the hepatitis delta virus. Eight distinct genotypes, with different geographical distribution, have been identified[167]. Its genome consists of a single-stranded negative circular RNA molecule ~1.7 kb long, and encodes only the hepatitis delta antigen (HDAg, distinct in HDAg-S, small, and HDAg-L, large), which together with viral RNA form the HDV ribonucleoprotein[170,171]. HDV exploits the host RNA polymerase II for the replication of its genome. HDV envelope is formed by the S, M, and L proteins of HBV, and the latter is necessary for HDV to enter the host cell as well as in the assembly and exit phases[171]. However, replication within the hepatocyte can occur in the absence of HBV components, and it is thought that in addition to the extracellular propagation pathway, HDV can also spread by a cell-division mediated pathway[172]. This virus has the same modes of transmission as HBV (parenteral, inapparent parenteral, sexual, and rarely vertical)[168,171].

Only HBV infected people can contract delta hepatitis. Coinfection that occurs when HBV and HDV are acquired simultaneously usually manifests itself as an acute hepatitis B, with varying severity up to fulminant hepatitis and has a chronicization rate of 2%-5% in adults. Superinfection refers to an HDV infection in a chronically HBV infected person. In this case, there is a higher risk of severe acute hepatitis and the probability of chronicity is about 80%-90%[168,171-173]. Chronic hepatitis D evolves rapidly towards cirrhosis and its complications[173]. The diagnosis consists in the detection of anti-HDV IgM or IgG and HDV RNA in the serum[168,171]. Current guidelines recommend looking for this infection systematically in HBV infected patients (European Association for the Study of the Liver, Asian-Pacific Association for

the Study of the Liver) or preferentially in certain categories of people (American Association for the Study of Liver Diseases).

Until recently, there were no specific approved therapies for hepatitis D. The latest international guidelines recommend using peg-IFNα to treat this infection (in association with NAs if patients have high HBV DNA levels). This drug demonstrated HDV RNA suppression in 19%-57% of treated patients at the end of the treatment but is characterized by high rates of post-therapy relapse[169]. In addition to the well-known side effects, it should not be used in patients with decompensated disease. Recently, 24 wk of peg-IFN-lambda showed similar antiviral effect but better tolerability compared to peg-IFNα[174].

Bulevirtide acetate (Hepcludex®, previously known as Myrcludex B, MyrB) was approved in 2020 in the European Union for the treatment of chronic HDV infection in adults with compensated liver disease[175]. The recommended dosage is 2 mg s.c. once daily, but the optimal duration of therapy is currently not specified. This drug blocks the entry of HBV and HDV into cells by binding sodium-taurocholate cotransporting polypeptide[175]. The results of a phase 2b clinical trial with primary endpoint HDV RNA reduction by 2 log or HDV negativity showed that 24 wk of treatment with MyrB in combination with TDF allowed patients to reach the endpoint in a significantly higher percentage of cases compared to those on TDF monotherapy (46.4% in the MyrB at 2 mg/die plus TDF arm *vs* 3.3% in the TDF in mono one) and that HDV RNA was suppressed in a dose-dependent manner. Twelve weeks after the end of therapy, however, the relapse was a frequent event[176]. Currently, some studies on bulevirtide are evaluating mono or combination therapies, different dosages, and durations, and the effect on patients with liver disease of varied severity.

Prenylation inhibitors block the prenylation of HDAg-L mediated by farnesyl transferase, preventing the bond of HDAg-L to HBsAg. Oral lonafarnib (LNF) inhibits the farnesyl transferase and makes it impossible for HDV particles to form. It reduced HDV RNA significantly more than the placebo in a study where it was used for 28 d; this therapy was associated with the appearance of some side effects, including gastrointestinal that were the most frequent[177]. Subsequently, LNF was tested in combination with ritonavir (ritonavir inhibits the CYP3A4 that metabolizes LNF, allowing the LNF dosage to be lowered), peg-IFNα, or lambda. The combination peg-IFN-lambda/LNF/ritonavir taken for 24 wk recently showed that it lowered HDV RNA > 2 log in most (25/26) treated chronic HDV patients and led to undetectable or "below the lower limit of quantification" HDV RNA in more than 50% of them at the end of treatment[178].

Among HBsAg secretion inhibitors, there is REP 2139, which in combination with peg-IFNα 2α (REP 301 study) was safe and was associated with HBsAg suppression in 5/12 patients and HDV RNA control in 7/12 patients after 1 year from the end of treatment[179]. This protocol showed persistence of the results in terms of safety and virological control of HBV and HDV over an extended follow-up period of 3.5 years [180].

Liver transplant is a therapeutic option for HDV patients who have not benefited or cannot use medical therapy[169].

HEPATITIS A

HAV is a single-strand RNA virus belonging to the Hepatovirus genus of the Picornaviridae family that was discovered by Feinstone *et al*[181] in 1973. HAV is able to withstand moderate temperatures and to survive in the environment. Therefore, it is transmitted through the orofecal route[182]. The clinical manifestations of HAV infection depend on the age at which the infection is contracted. In children, it occurs in less than 30% of cases with symptomatic hepatitis, while 80% of infected adults have a severe hepatitis with jaundice and a significant increase in transaminases[183].

EPIDEMIOLOGY

HAV infection is widespread worldwide, with a higher prevalence in low- and middle-income countries[184] where the hygienic-sanitary conditions are scarce. Most of the children (90%) contract the infection before the age of 10, often without clinical manifestations. Epidemics rarely occur as older children and adults are generally immune. Intermediate endemicity is observed in North Africa, Eastern Europe, the Middle East, Latin America, and middle-income regions in Asia[185]. High-income

countries such as the United States and West European countries have lower rates of HAV infection, but most of their adult population is not immune to this virus, resulting in a higher probability for this category of developing clinical manifestations due to HAV infection than in the low-income countries[186]. In areas with high endemic and poor hygienic and sanitary conditions, HAV infection is transmitted mainly by contaminated water. In intermediate endemic areas HAV is mainly transmitted through contaminated food and water[187], while in non-endemic countries, risk factors are travel to endemic areas and oroanal sex[188,189].

CLINICAL MANIFESTATIONS

Clinical manifestations of HAV infection range from the total absence of signs and symptoms to acute hepatitis. Acute liver failure (ALF) is also possible, but it is a rare event. This infection usually resolves itself spontaneously and is not responsible for chronic hepatitis. After a period of 2-7 wk from the time of infection, the patient may develop typical symptoms of acute hepatitis, such as jaundice, asthenia, vomiting, dark urine, clay-colored stools, fever, or general malaise[190]. Blood tests show a severe increase in transaminases (also 100 times the upper limit of normal), usually with ALT > aspartate aminotransferase, increased total bilirubin, and increased alkaline phosphatase. It is important to monitor PT and the onset of portosystemic encephalopathy to assess the presence of ALF[191], although this is a very rare occurrence (less than 1%)[192]. Atypical manifestations may also be present, including relapse hepatitis, autoimmune hepatitis, prolonged cholestasis, and acute kidney failure[193]. Considering that the clinical presentation of hepatitis by HAV is similar to any other hepatitis, the diagnosis of HAV infection requires the dosage of IgM anti-HAV. IgM anti-HAV becomes detectable in the blood about 5 d after the onset of the disease and remains positive for a short period after healing. The recent execution of the HAV vaccine can determine a positive test result for IgM anti-HAV. The presence of IgG anti-HAV indicates a previous HAV infection[194].

THERAPY AND PREVENTION

There is no specific cure for HAV acute hepatitis. Therefore, supportive therapy is indicated, trying to alleviate the symptoms of the infection (fever, nausea, vomiting) and intervening on any other complication (dehydration, electrolytic abnormalities, *etc.*). IFN has been evaluated in the past for the treatment of hepatitis A. This drug has been shown to reduce viral replication in cell cultures[195] and has been administered in sporadic cases of patients with severe or fulminant hepatitis secondary to HAV infection[196]. Sofosbuvir showed the ability to inhibit replication of HAV *in vitro* [197], but there are no reported cases in literature where this drug was administered *in vivo*. Viral hepatitis are responsible for 12% of acute liver failure, and of these only in 0.35% of cases the causative agent is HAV[198]. HAV infection is the main cause of ALF in the pediatric population in some countries[199-201]. Liver transplantation is necessary in 30% of ALF due to HAV infection, therefore it is a rare indication[198]. In some case reports, the effectiveness of plasma exchange in ALF therapy due to HAV infection is reported[202,203], while the use of corticosteroids in a small cohort of children was associated with reduced hepatocyte destruction and with higher survival rates compared to controls[204]. N-acetyl cysteine, although very effective in ALF secondary to paracetamol abuse, does not appear to provide any benefit in the treatment of HAV related ALF[205]. In the case of worsening liver function, liver transplantation is required[206]. Liver transplantation in ALF secondary to HAV infection is burdened with a high mortality[207] and is rarely complicated even by a post-transplant HAV reinfection[208].

The improvement of sanitary conditions and vaccination play a fundamental role in the prevention of HAV infection[209,210]. Available vaccine formulations for hepatitis A (inactivated and attenuated) have proven effective and safe in immunizing in large trials involving nearly 750000 patients worldwide[211]. The vaccine can be administered in one or two doses 6 mo apart to obtain long-term immunization[212]. Lower protection rates following vaccination are observed in immunosuppressed patients, (HIV-infected patients, transplant recipients) and in elderly people[213,214]. The administration of the vaccine is particularly recommended in patients with concomitant chronic HCV and/or HBV infection[215] and in non-immune people traveling in areas of high endemia[216,217]. The vaccine may also be administered in

secondary prophylaxis, single-dose, 14 d after contact, after the exposure to a patient with known HAV infection, showing the same efficacy as immunoglobulins[218].

HEPATITIS E

Hepatitis E, the liver disease caused by HEV, is a health problem that is receiving increasing attention over the years, although many aspects concerning this infection are still lacking. It has a self-limiting and benign course in most people, but some categories are particularly at risk of serious outcomes, such as pregnant women who have a mortality risk of about 25%, immunocompromised patients who can develop a chronic hepatitis (and its consequences), and patients with previous liver disease who are at risk of acute-on-chronic liver failure.

EPIDEMIOLOGY

The strong negative impact of HEV on human health can be deduced from data estimating that in 2005 there were about 20.1 million cases of hepatitis E (genotypes 1 and 2) infections and 70000 deaths worldwide[219]. A recent study[220] estimated that more than 900 million people have had contact with this virus; moreover, in many regions of the world HEV is the leading cause of acute viral hepatitis[221]. While in some countries HEV infections appear to have remained stable, in others, including European ones, there has been an increase over time (data probably explained also by an increase in identifications)[221,222]. However, the real burden of hepatitis E on human health is not yet fully known[220].

The transmission mode and diffusion of HEV infection vary greatly depending on the countries, and in the same country there are some epidemiological differences depending on the areas (*e.g.*, southwest compared to other regions in France)[221,223]. The most frequent transmission mode in areas of the world with poor hygiene standards is the oral-fecal route, mediated by water (genotypes 1 and 2), while in developed countries is zoonosis (genotypes 3 and 4)[223].

DISCOVERY AND VIROLOGY

The existence of an enterically transmitted non-A, non-B hepatitis virus was first hypothesized in 1978 by Khuroo[224], while identification under microscope occurred in 1983. The name "hepatitis E virus" was proposed for the first time in 1988[225].

HEV is a non- or quasi-enveloped icosahedral virus, 27-34 nm in size that contains a single strand, positive-sense RNA 7.2 kb long. The quasi-enveloped variant that exists in circulation is due to the lipid membrane originating from infected cells. The HEV RNA genome contains three ORF: ORF1 encodes a polyprotein that includes an RNA-dependent RNA polymerase; ORF2 encodes the capsid protein; and ORF3 encodes a protein involved in the viral egress[221,225]. ORF 4, recently described for HEV-1, has a supporting role for viral polymerase[226]. Despite the advances in knowledge of this virus, several aspects of the HEV life cycle are not yet known, including the exact mechanism by which the virus enters the host's cells (*e.g.*, entry receptors), some aspects of the relationship between host components and virus, and many processes concerning assembly and cell exit[227].

It is likely that, as well as other major hepatotropic viruses, HEV causes liver damage mainly through an immune-mediated mechanism[228]. Briefly, the host response starts with an initial phase in which the innate immune system detects the virus through pathogen recognition receptors such as TLR3, TLR7, RIG-I, and MDA5 [229,230]. Subsequently, the production of IFN and other cytokines or chemokines that amplify the response against HEV take part[229,230]. Liver migration and activation of innate cells such as natural killer and neutrophils and then recruitment of T lymphocytes determine the antiviral response and contextually the immune-mediated liver injury[228,231]. The immune response (including antibodies) against specific regions of the ORF2 protein is critical in the fight against the virus. Although the HEV can hinder some steps of both innate and adaptive responses[228,229,232], the combination of these events usually leads to the clearance of the virus. Anyhow, the role of anti-HEV antibodies in the resolution of the infection[232] as well as the specific role of other elements of the immune system in the host-HEV relationship is not yet

fully understood.

DIFFERENT GENOTYPES AND TRANSMISSION ROUTES

HEV is part of the Herpesviridae family that is divided into two genera, Orthohepevirus and Piscihepevirus. The genus Orthohepevirus includes four species (A-D), while the genus Piscihepevirus has only the Piscihepevirus A species. Eight distinct genotypes belong to the Orthohepevirus A species, and some viral subtypes of these genotypes have been recognized[225,233].

Orthohepevirus A genotypes 1, 2, 3, and 4 (HEV-1, HEV-2, HEV-3, and HEV-4) infect humans differently from the other genotypes of this species[225,226]. However, a case of a middle-aged liver transplanted Somalian man who regularly consumed camelid meat and milk infected with dromedary HEV (HEV-7) was also described [234].

HEV-1 and HEV-2 are restricted to higher primates (humans and several species of monkeys), but the finding of HEV-1 in the serum of Egyptian horses suggested that in rare circumstances transmission of these genotypes to other animals is also possible [233].

HEV-1 and HEV-2 spread among the human population mainly by the oral-fecal route that consists in ingestion of water contaminated with human feces[221,223,225, 235]. This phenomenon typically occurs in regions of the developing world with poor hygiene standards, such as those in which untreated water, for example of a river, is drunk or used for cooking, bathing, and waste disposal. In such contexts, climatic changes (*e.g.*, heavy rainfall or monsoons) can increase the risk of HEV infection spreading[235,236]. These genotypes are responsible for the occurrence of epidemics and outbreaks. The first one to be retrospectively documented broke out in Delhi (India) in 1955[225]. Today HEV infection is considered hyperendemic and associated with the development of outbreaks in various parts of north, east, and west Africa, south and central Asia, the Middle East, and Latin America[223,236]. In such areas, a high percentage of inhabitants were found to be anti-HEV IgG positive[220]. The typical route of transmission of these genotypes makes it conceivable that an improvement in hygiene conditions could result in a containment of the spread of infection[221,225]. In addition to the orofecal route, vertical *via* blood transfusion and person-to-person transmissions were documented[225,236]. In developed countries, where a better level of sanitation is guaranteed, HEV infection is sporadic and it is acquired as zoonoses, by travelling to endemic areas, or by blood transfusion[221,225].

HEV-3 and HEV-4 infect animals (including pigs that are the main reservoir and source of contagion for humans, wild boars, and deer) and humans and are responsible for the zoonotic form of the disease. They can be transmitted to humans in several ways: consumption of undercooked or raw meat (a recurring example in literature of a source of infection are the Corsican sausages "figatelli" that contain pork liver) or milk from infected animals; consumption of fruits, vegetables (strawberries, raspberries, lettuce, *etc.*), or seafood contaminated with feces of infected animals, and direct contact with infected animals (farmers, veterinarians, slaughterhouse workers) [221,223,225,236]. Today it is known that in addition to the most often mentioned animals (pig, wild boar, and deer), many others can serve as reservoirs for HEV-3 and HEV-4 (*e.g.*, rabbits, cattle, rats, goats, sheep, dogs, horses, Java mongoose, dolphins, clouded leopard, Asiatic black bear, and others)[233]. An aspect not yet well known is which animals are "true hosts" for HEV and which others host it transiently. Blood transmission is also possible for HEV-3 and HEV-4[221].

The other Orthohepevirus A genotypes were identified in animals: HEV-5 and HEV-6 in wild boar, HEV-7 and HEV-8 in camelidae (dromedary and bactrian camel, respectively)[233].

Orthohepevirus B, of which four genotypes are known, has been isolated mainly in chickens but also in turkeys, ducks, and other birds. However, the possibility that some of its strains can infect mammals cannot be ruled out[233]. In the Orthohepevirus C species there are two different genotypes (HEV-C1, HEV-C2), whose presence has been documented in different types of rodents (rats, mice, shrews, ferrets, minks, and others). Some cases of human infections from rat HEV have been recently discovered in patients from Hong Kong and in a man suffering from acute hepatitis, who probably contracted the infection in central Africa[233,237,238]. Orthohepevirus D species was isolated in the bat. The Piscihepevirus genus includes only the Piscihepevirus A species that infects the cutthroat trout and other fish[233]. An illustration of the link between HEV genotypes, hosts, and routes of transmission is provided in Figure 3.

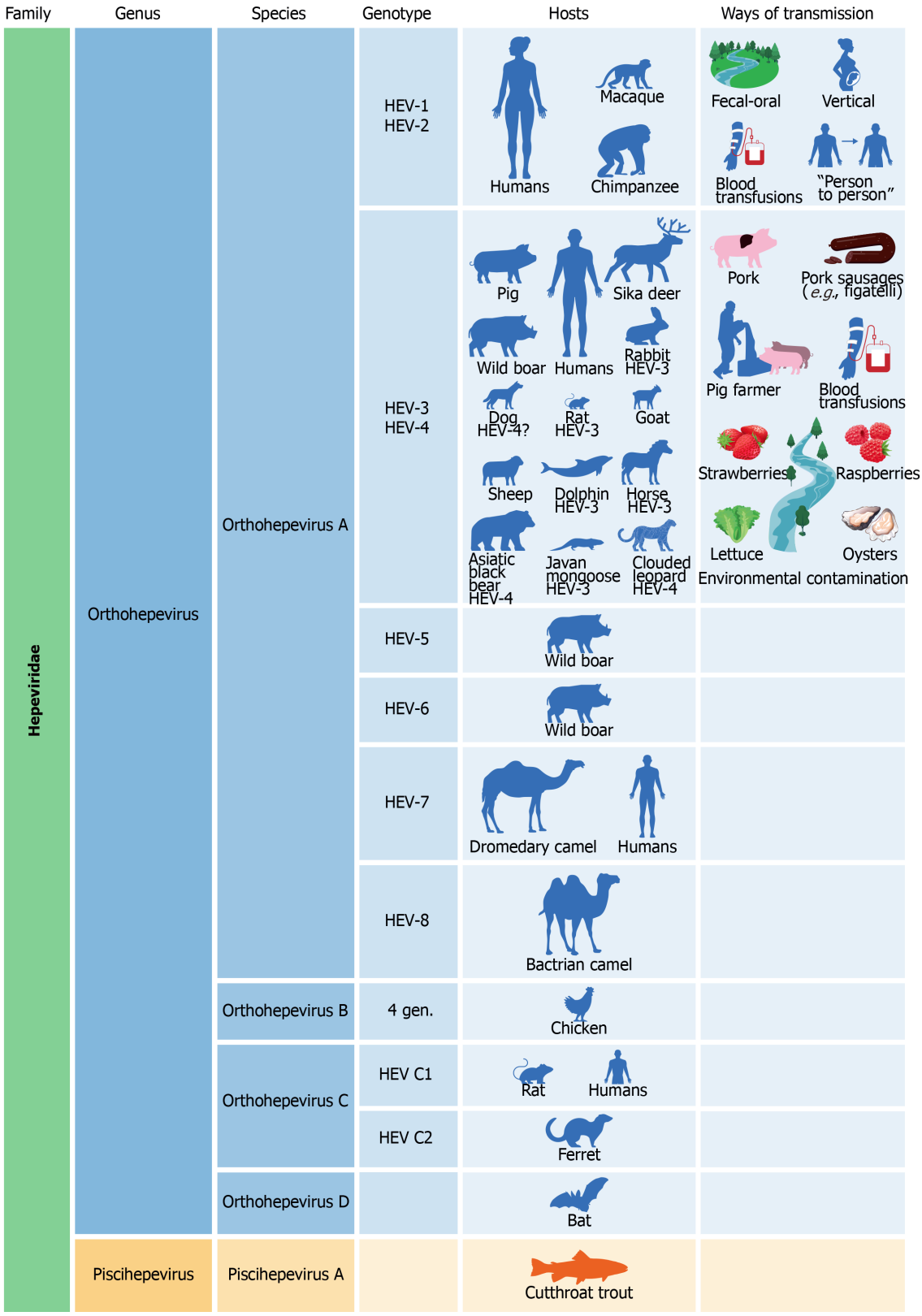


Figure 3 Classification, hosts, and ways of transmission of the hepatitis E virus. HEV: Hepatitis E virus.

DIAGNOSTIC TESTS

Anti-HEV IgM appear about 4 wk after infection, approximately with the onset of symptoms, and their persistence in the serum is variable (they usually last 3 or 4 mo, but cases in which they were detectable after a longer time were also described). Given their kinetics, they reveal a recent infection and can be considered a marker of acute hepatitis E[221,239,240]. In addition to anti-HEV IgM, anti-HEV IgA antibodies are

considered markers of acute hepatitis E, but it is not a widely used test[221].

Conversely, anti-HEV IgG appear after anti-HEV IgM and last several years. They are used to diagnose a past infection and are indispensable for seroprevalence studies of such disease. Because anti-HEV IgG appear quite early in the course of the disease they can be detected together with IgM in the acute phase; in this way they help to reduce the misdiagnosis rate that occurs if IgM are considered alone[221,240]. The antibodies against HEV are determined by enzyme immunoassays[239,240]. For diagnostic purposes, due to the non-optimal reliability of antibody assays, guidelines of the European Association for the Study of the Liver recommend using the anti-HEV IgM (or anti-HEV IgG) in combination with the HEV RNA, or antibodies in combination with each other, or HEV RNA (in blood or stool) alone to reveal a current infection[221].

HEV RNA is detectable soon after infection before symptoms appear and generally lasts a maximum of 6 wk; it confirms the diagnosis of infection. To improve comparability of nucleic acid amplification test results and ensure a correct genotype identification, the WHO defined international standard and an international reference panel for HEV genotypes 1-4. Another direct test consists of the search for the HEV capsid antigen, but its role in the diagnosis is still unclear[221,240]. Currently it is difficult to know how widespread screening is for HEV, but the raising awareness of the spread of hepatitis E even in high-income countries over the years will likely result in a reduction of undiagnosed cases in the near future.

CLINICAL MANIFESTATIONS

After HEV contact, the incubation period is about 2-6 wk, but it can be very variable. HEV infection is generally asymptomatic or with very few symptoms and self-limiting. This aspect also contributes to the non-definitive knowledge on the real diffusion of the disease. In about 5%-30% such infection is symptomatic and has a similar course to that of other acute hepatitis. In these cases, the infection is responsible for nonspecific symptoms, such as asthenia, nausea, vomiting, fever, muscle, and joint pains and then jaundice, itching, dark urine, and light-colored stools. Contextually, blood tests can show alterations in liver biochemistry such as high transaminases (ALT often exceeds 1000 IU/L), bilirubin, gamma glutamyl transferase, and alkaline phosphatase. It was observed that HEV-3 and HEV-4 tend to be associated with clinically more severe hepatitis in older males, but the underlying reasons are still unclear. Rarely can HEV infection result in ALF[219,223,225,239,240].

While healthy, immunocompetent people usually have a benign course, there are some categories for whom HEV is more dangerous. Patients with pre-existing chronic liver disease can develop acute-on-chronic liver failure due to hepatitis E virus infection[223,226]. Nowadays it is estimated that many cases of liver damage of undetermined etiology or suspected drug origin are caused by HEV. For this reason, it is recommended to test patients with liver biochemistry compatible with hepatitis or diagnosed with ALF or acute-on-chronic liver failure of still undetermined etiology for HEV infection in addition to other causes of liver injury.

Immunosuppressed patients, including organ or stem-cell transplant recipients, HIV infected patients, patients receiving chemotherapy for hematological diseases, or long-term therapy for autoimmune disorders are another category at risk of worse outcomes[221,226,240-242]. In these patients the risk of developing chronic hepatitis E is consistent, given the inability of their immune system to eliminate the virus. Studies on organ transplant patients infected with HEV showed a chronicization rate of more than 60%[243]. In this group, it is debated whether to consider "chronic" an infection from HEV when the RNA has been identifiable for at least 6 mo, or if it is detectable for more than 3 mo after infection[221,244]. Chronic HEV infection has been described mainly for genotype 3, but chronic infections from HEV-4 were also reported, and one case of chronic infection by HEV-7 (as reported above) was described.

Some cases of chronic hepatitis E by HEV-1 have recently been claimed[245,246]. Chronic hepatitis E generally has a slightly symptomatic or asymptomatic course, with only mild alterations of liver biochemistry[240,241,243]. Moreover, anti-HEV antibodies can often give false negative results, and therefore the search for HEV RNA is particularly useful in these people[240]. This form can progress through the development of increasingly severe fibrosis up to decompensated cirrhosis. Liver injury, as has already been observed for other viral hepatitis, can regress after the elimination of the virus[221,240].

HEV-1 and HEV-2 are responsible for a high percentage of liver failure and mortality (25%-30%) in pregnant women during the third trimester, and HEV infection during pregnancy was also associated with increased neonatal morbidity (*e.g.*, preterm birth) and mortality[223,247]. Several factors have been hypothesized to explain the high severity of HEV infection in pregnancy, including host (hormonal and immune changes) and virus-related factors, but the exact causes are not known[247]. Another noteworthy aspect is that there are considerable geographical differences regarding the severity of the course of HEV infection in pregnancy[223].

The hepatitis E virus is responsible for several extrahepatic manifestations. The pathophysiology of these events is not yet fully understood, but direct damage by the virus is not excluded[221,226,240]. It has been observed that HEV can replicate in many other locations besides the liver, and these findings raised the hypothesis that the entry receptors of HEV are not limited to hepatocytes. Among the extrahepatic manifestations there are neurological disorders (Guillain–Barré syndrome, neuralgic amyotrophy, Bell's palsy, meningoencephalitis, peripheral neuropathy, mononeuritis multiplex), kidney injury (membranoproliferative and membranous glomerulonephritis, IgA nephropathy), hematologic disturbances (thrombocytopenia, hemolysis, aplastic anemia, monoclonal immunoglobulin, cryoglobulinemia), acute pancreatitis, and some reports of thyroiditis, arthritis, and myocarditis[221,240].

THERAPY AND VACCINE

Antiviral therapy is usually not necessary for acute hepatitis E, and in any case, there are no specific or extensively tested treatments. In cases of severe forms of hepatitis E and liver failure, RBV was successfully used and resulted in improvement of liver biochemistry and clearance of HEV RNA; corticosteroids have also been shown to be effective in some patients[221,240].

For patients diagnosed with a chronic form of hepatitis E in the context of transplant recipients, it is recommended to reduce immunosuppressive therapy as a first step. This measure alone proved to cause HEV clearance in a consistent percentage of patients (nearly one-third)[221,243]. Currently, RBV represents the therapy of choice for chronic hepatitis E, but its mechanism of action against this virus remains unknown. In a retrospective study on 59 organ transplant patients suffering from chronic hepatitis E treated with RBV, the SVR rate was 78% after a first course, and 85% if re-treatments are considered[248]. In another study of 255 organ transplant patients with chronic HEV infection, 81.2% of them reached SVR after the first course [249]. In these studies, RBV was used with a median dose of 600 mg/d for a median duration of 3 mo, and this is the most used protocol. As known, RBV treatment can be associated with the onset of side effects including hemolytic anemia, skin reactions, or a dry cough. Furthermore, due to its teratogenic potential, the use of RBV is not recommended in pregnancy, and for this category careful monitoring and supportive care must be provided; therefore, prevention of infection is a key point[221,247].

Peg-IFNα is another drug used for hepatitis E in liver transplanted patients and people undergoing hemodialysis, but it is contraindicated in kidney, heart, lung, and pancreas transplant recipients due to the risk of rejection[221,240]. In cases of non-response or intolerance to RBV and the patient is not eligible for IFN, there are no options to continue therapy[221,249,250]. A recent study in which sofosbuvir in monotherapy was used in nine chronic hepatitis E patients showed the ineffectiveness of this drug in eliminating HEV[250]. For these reasons, there is a need for new effective drugs with specific anti-HEV activity[250,251].

An HEV vaccine (Hecolin®) has been available in China since 2011; this vaccine was designed based on the ORF2 region of HEV-1 and showed very high efficacy in preventing symptomatic hepatitis E in a randomized placebo-controlled trial[252]. It proved to be effective for several years, but the overall duration of protection is yet to be known. The vaccine looks safe in pregnant women, but further concrete data is needed. The other open questions are efficacy and safety in specific categories of patients (such as those suffering from chronic liver diseases) and effectiveness against other genotypes[221].

CONCLUSION

In conclusion, although viral hepatitis still represents a major public health problem and a leading cause of mortality, important milestones have been achieved in this

field. Nowadays, hepatitis C is curable in almost all patients by simple, safe, and short-course therapies. To eradicate it, it is crucial to focus on case-finding and linkage to care initiatives. The scenario of hepatitis B and D is changing, with a reduction in their prevalence largely due to the anti-HBV vaccination. Currently available anti-HBV therapies are effective in suppressing viral replication, but they do not eradicate HBV. However, several new therapeutic approaches are being studied for HBV and HDV infections, and in 2020 an entry inhibitor for the treatment of chronic hepatitis D was approved in the European Union. Improvements in hygiene conditions and vaccination can prevent hepatitis caused by HAV and HEV, but the vaccine against the latter virus is not available in all countries. Hepatitis E still represents a challenge as its pathophysiology and real spread are not fully known. Moreover, currently available treatment options are not feasible in all patients.

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MINIREVIEWS

Addition of statins to the standard treatment in patients with cirrhosis: Safety and efficacy

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Abstract

This review summarizes the safety and efficacy of statins in patients with cirrhosis. Due to concerns about the safety of statins in patients with impaired liver function, they have recently been investigated as a potential treatment option in cirrhosis. The most clinically significant adverse event is statin-related myopathy, and this may be related to the high serum statin concentrations in the setting of severely impaired liver function. Rhabdomyolysis is the most serious and potentially life-threatening manifestation. It has recently been demonstrated that the recommended dose of simvastatin in patients with decompensated cirrhosis would be 20 mg/d because higher values, such as 40 mg/d, are associated with many adverse events, especially muscle injury. Likewise, simvastatin should not be administered to patients with Model for End-stage Liver Disease score > 12 and/or Child-Pugh class C because of the high risk of severe muscle injury. Due to the pleiotropic effects, the focus on statins has shifted from being considered harmful to something useful. Through these effects, statins could prevent liver-related morbidity and mortality in cirrhotic patients. Observational studies in large populations of patients with cirrhosis have shown that treatment with statins to decrease high cholesterol levels was associated with a reduced risk of hepatic decompensation, hepatocellular carcinoma development and death.

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The few randomized controlled trials in patients with cirrhosis and portal hypertension showed that statins lower portal pressure, quite likely through a reduction in hepatic resistance. Another large randomized controlled trial in patients with variceal bleeding showed that simvastatin in addition to standard of care did not prevent rebleeding but improved survival rate. Despite these encouraging outcomes, the quality of the evidence regarding the use of statins is low or very low due to the observational characteristics of most of the studies involved. Therefore, it is advisable to perform further randomized controlled trials on a large series of patients with hard clinical endpoints, using different statin types and varying doses. The objectives would be to prevent liver-related morbidity and mortality rather than treating cirrhosis complications to take additional information that makes it possible to add statins to the standard of care of these patients.

Key Words: Cirrhosis; Liver disease; Statins; Safety; Efficiency

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Core Tip: Several observational studies of statins in patients with liver disease were published, but they were highly biased. On the other hand, only one randomized controlled trial improved survival in Child-Pugh class A/B patients but did not reduce variceal rebleeding. Finally, the dose and type of statin to be used should be defined. This review concludes that new randomized controlled trials are necessary before endorsing statin use in patients with chronic liver disease.

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INTRODUCTION

The 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors, "statins," have been used for more than three decades to treat the increase of serum cholesterol by inhibiting its synthesis *via* the competitive inhibition of the hepatic enzyme HMG-CoA reductase [1]. They represent a breakthrough in the treatment and prevention of atherosclerotic cardiovascular disease. High-quality evidence from large randomized controlled trials (RCTs) supports the use of statins in primary and secondary prevention of atherosclerotic cardiovascular disease[2-5]. Nevertheless, no patients with liver disease were included in these trials, and consequently there is limited data regarding the therapeutical role of statins over this population.

Atherosclerosis has been widely recognized as an inflammatory process[6]. Statins prevent inflammatory atherosclerotic cardiovascular disease by two mechanisms: the first one is direct and acts by reducing the plasma cholesterol levels, and the second one is indirect and independent of cholesterol levels, also known as the pleiotropic effect. This effect reduces atherosclerotic inflammation independently of the serum cholesterol levels and any cholesterol levels variation[7,8]. The isoprenoid intermediates, such as farnesyl-PP and geranylgeranyl-PP, are essential in the biosynthetic pathway of cholesterol. These isoprenoid intermediates lead to isoprenylation, adding lipophilic molecules to various proteins, including the small guanosine triphosphate binding proteins Rho, Ras, and Rac (also known as small GTPases), which are activated and translocated from the cytoplasm to the cellular membrane. The inhibition of isoprenylation of Rho, Ras, and Rac by statins keeps them inactive in the cytoplasm, as shown in Supplementary Figures 1 and 2. The inhibition of isoprenoid intermediates favors the pleiotropic effects of statins by preventing small GTPase protein isoprenylation, implicated in intracellular signaling pathways[8].

Moreover, the activity of Rho kinase is reduced by the inhibiting effect of statins upon both hepatic stellate cells (HSC) and liver sinusoidal endothelial cells[9]. In experimental models, these effects seem to reduce collagen production and the activation of HSC in early fibrosis and proliferation, cytokine production and contraction of activated HSCs in cirrhosis[9,10]. Additionally, statins might decrease the turnover of HSCs inducing the senescence of these high activity cells[9-11]. On the other hand, in cirrhotic livers, statins improve endothelial dysfunction by the upregulated activity of endothelial nitric oxide synthase and nitric oxide availability and lower portal pressure[9,12]. Krüppel like factor 2 mediates statin-induced upregulation of endothelial nitric oxide synthase to decrease HSC activation and produce a further decrease of portal pressure[13,14]. These data deliver the rationale for statin use in patients with cirrhosis and portal hypertension[15,16].

This review will discuss available evidence about the safety and efficacy of statins in patients with cirrhosis.

SAFETY

The most commonly reported adverse events during treatment with statins are relatively mild and often described as transient gastrointestinal symptoms (diarrhea, abdominal pain, flatulence and constipation), headache and rash[17]. Muscle injury, liver injury and new-onset diabetes mellitus are proven adverse events of statin therapy[18]. Statin plasma levels have been regarded as an index of these potential adverse events[19], and they depend on several factors[20]:

(1) Statin dose: High-intensity statin therapy reduces cardiovascular events more than lower intensity equivalent treatment, but the safety impact of using the first one is unknown. On that subject, a meta-analysis showed that high-intensity statin therapy augmented the incidence of increased aminotransferase levels [relative risk = 3.10; 95% confidence interval (CI): 0.88-7.85] compared with lower intensity statin therapy. Based on clinical trials, the authors conclude that an aggressive statin therapy increases aminotransferase levels more than a lower intensity one[21].

(2) Statin type: In a rat model, Nezasa *et al*[22] showed that rosuvastatin was taken up by hepatocytes more selectively and more efficiently than simvastatin. A possible reason for this finding is that rosuvastatin requires an anion transporter to enter into the cells in the same way as pravastatin, another hydrophilic statin. On the other hand, simvastatin does so by passive diffusion. This possibly happens due to its greater lipophilicity, and in this way, it is similarly taken up by the liver and by extrahepatic tissues as other lipophilic statins do, such as atorvastatin and fluvastatin. These different mechanisms of entry into the cells may be consistent with specific organ toxicity.

In the meta-analysis mentioned above, when evaluating the different effects of hydrophilic and lipophilic statins were studied, higher intensity therapies based on the first ones showed an increased risk for raising aminotransferases levels, whereas those using lipophilic statins did not. Regarding creatine kinase (CK) levels, these were not increased during evaluating hydrophilic statins, whereas lipophilic statins revealed an increased risk with higher intensity treatment[21]. Therefore, the increase in aminotransferase levels is likely to be more troublesome when high doses of hydrophilic statins are prescribed, whereas CK elevations become problematic with equally aggressive lipophilic statin treatment.

(3) Drug-drug interactions: Drug interactions that involve statins may be enhanced in the setting of cirrhosis, given the altered statin pharmacokinetics[23]. In this sense, the total body clearance of statins is very high due to the important hepatic first-pass effect. Except for pravastatin metabolized enzymatically in the liver cytosol, all statins undergo extensive microsomal metabolism by the cytochrome P450 isoenzyme systems[20]. The cytochrome 4503A4 isoform commonly has a competitive inhibition with drugs at the enzymatic level and may alter statin disposition, leading to increased plasma levels and greater risk of adverse events[24]. Since 2011, the United States Food and Drug Administration is assuming a more significant role in post-marketing surveillance and keeping package labeling of statins with current contemporary literature[25]. In Supplementary Table 1 are shown simvastatin-drug interactions[26]. This information is deemed incomplete, therefore an updated bibliography and other tools for evaluating the interaction with each drug are needed. It is important to note that there are no simvastatin interactions with drugs commonly used in cirrhosis, such as non-selective β-blockers (NSBBs), diuretics, quinolones, rifaximin and anticoagulants.

Also, the consumption of grapefruit juice, at more than 1 quart per day (approximately 1 L/d), is known to inhibit cytochrome 3A4 isoenzyme activity in the enterocyte[27]. Likewise, gemfibrozil therapy reduces glucuronidation and consequently the elimination of statins[28]. Therefore, the effect of both could increase statin availability and in turn potentially raise their systemic exposure and side effect occurrence.

(4) Reduced statin metabolism and/or transport activities: It is well known that cirrhosis significantly affects statin pharmacokinetics[29]. However, although no study has so far evaluated the pharmacokinetics of simvastatin in cirrhotic patients, there are data with other statins in Child-Pugh (CTP) class A and class B patients[30-32]. So far, there are no reports about the pharmacokinetics of statins in CTP class C patients. It is known that the maximum plasma concentration increases with rosuvastatin, the area under the curve becomes larger with fluvastatin and both parameters increase with atorvastatin in cirrhosis[30-32]. Furthermore, another mechanism could be a single nucleotide polymorphism in SLCO1B1, which encodes the OATP1B1 that regulates the hepatic uptake of statins[33]. Recent studies have shown that a diminished expression of SLCO1B1 is associated with progressive alcoholic liver disease[34,35]. This last mechanism reduces the hepatic uptake and increases the blood levels of statins and enhances the risk of adverse events[19].

Proven adverse events

Muscle injury: Statins have been associated with a broad spectrum of muscle injury ranging from asymptomatic serum CK elevations to rhabdomyolysis[36]. Statin-associated muscle adverse events ranged from 1% to 5% in controlled clinical trials and 11% to 29% in observational ones[36]. Much of the concern about statin's potential side effects arise from the results of these observational studies.

There are well-established risk factors for statin-related muscle injury[36]. Mounting evidence suggests that higher-dose statin therapy confers a greater risk of muscle injury (see Safety, Statin dose). Statin-related muscle injury is also related to interactions between drugs that increase serum statin levels (see Safety, Drug-drug interactions).

Many patients experience the onset of symptoms in a temporary relationship when starting statin therapy. These symptoms often carry patients and physicians to claim that statins are the cause for them, even when there are very few randomized data to support these assertions. The N-of-1 trial in a group of eight patients experiencing myalgia while being treated with statins demonstrated that data on subjective side effects from observational studies are heavily loaded with bias. Patients were randomly assigned to receive either placebo or the same statin; each patient served as their self-control, and statin and placebo were alternated between them. Pain and interference with life activity were assessed using a weekly visual analog scale. The authors observed no significant difference between statin and placebo in myalgia score in each patient, with five patients resuming statin therapy[37].

Muscle safety in patients with cirrhosis was assessed in three RCTs that evaluated the effect of simvastatin on portal pressure and gastrointestinal bleeding occurrence [16,38,39]. These studies included patients from all CTP classes, but the proportion of CTP class C patients was low. Abraldes *et al*[16] reported a 2-fold increase — or higher — in CK levels for 1 out of 29 placebo patients and 2 out of 30 simvastatin patients. They concluded that the simvastatin safety profile was excellent. Pollo-Flores *et al*[38] reported a 7% incidence of myalgia in the simvastatin group and a 10% in the placebo group. Finally, in BLEPS (Bleeding Prevention with Simvastatin) trial, when evaluating the addition of simvastatin to standard therapy for preventing variceal rebleeding, 2 out of 69 patients on simvastatin developed rhabdomyolysis (2.9%)[40], which was a concerning issue considering an incidence of 0.009% to 0.1% in the general population [41]. The authors observed that both patients had a more advanced liver disease with bilirubin levels greater than 5 mg/dL. Therefore, Abraldes *et al*[16] concluded that severely deteriorated liver function patients might develop muscle injury at lower doses than the general population.

The LIVERHOPE-SAFETY trial was a double-blind, randomized, placebo-controlled, phase 2 trial in patients with decompensated cirrhosis and moderate-to-severe liver failure from nine university hospitals in six European countries[42]. Patients older than 18 years with CTP class B or class C disease were eligible and randomly assigned to receive for 12 wk (in a 1:1:1 proportion) either: Simvastatin 40 mg/d plus rifaximin 1200 mg/d (SVT40 + RFX), simvastatin 20 mg/d plus rifaximin 1200 mg/d (SVT20 + RFX) or placebo of both drugs. Randomization was stratified according to CTP class (B *vs* C). The main endpoint was the appearance of liver or muscle injury, as defined by changes in aspartate aminotransferase (AST) and alanine aminotransferase

(ALT), alkaline phosphatase and CK. The full analysis set included 44 patients: 16 in the SVT40 + RFX group, 14 in the SVT20 + RFX group and 14 in the placebo group. Patients in the first group showed an increase in CK at the end of treatment compared with patients in the placebo group (1060 IU/L *vs* 106 IU/L, $P = 0.014$). It was also observed that there were no significant changes in CK levels in the second group *vs* the placebo group (106 IU/L *vs* 67 IU/L, $P = 0.992$). Three patients (19%) from the SVT40 + RFX group developed liver and muscle toxicity consistent with rhabdomyolysis. See Safety, Reduced activities in statins metabolism and/or transport.

Liver injury: An electronic PubMed search of reported statin hepatotoxicity cases identified 40 patients in 26 publications[43]. The small number of reported cases is a testimony of the supposed hepatic safety of statins given many recipes for this class of drugs, *e.g.*, 142 million in 2008 in the United States alone[44]. Of the 40 cases, 17 patients were men, and most in the sixth or seventh decade of life. The initial symptoms were jaundice, anorexia, nausea, abdominal pain, adynamia and pruritus, similar to acute hepatitis. Mortality due to statins was exceptionally infrequent because only two cases were reported[45,46]. The time elapsed since the drug administration and the development of hepatotoxicity is highly variable: between 5 d and 4 years. However, in more than half of the cases, it occurred within 4 mo from the start of therapy. The interval between the discontinuation of the statin and drug-induced liver injury resolution ranges from a few weeks to 6 mo. The most frequent liver damage pattern is hepatocellular; however, a mixed pattern was also reported, with prolonged symptomatic cholestasis[47].

Based on this data, the Food and Drug Administration in 2012 issued a report on significant changes in the safety of the statin packet insert; all statins currently marketed appear to be associated with a shallow risk of severe hepatic injury[48]. Furthermore, continuous periodic monitoring with ALT does not help detect or prevent drug-induced liver injury and removes the need to perform it. Packet inserts now recommend performing liver function tests before starting statin therapy and then when clinically indicated.

Three RCTs that appraised the effect of simvastatin on portal pressure and gastrointestinal bleeding frequency assessed the liver safety of statins in patients with cirrhosis[16,38,40]. Pollo-Flores *et al*[38] did not report any patient with liver injury. Abraldes *et al*[16] observed a patient with aminotransferase increase in each trial. In 2009, with AST > 2 times the upper limit of the normal, simvastatin was continued, whereas in 2016, with ALT > 3 times the upper limit of the normal, simvastatin was stopped[40].

In the LIVERHOPE-SAFETY trial, due to advice from the data safety monitoring board, the SVT40 + RFX group was stopped prematurely when the first 10 patients completed treatment because of severe liver adverse events (grade 3)[42]. Patients within the SVT40 + RFX group showed a significant increase in AST and ALT than the placebo group (AST 191 IU/L *vs* AST 62 IU/L, $P = 0.0009$ and ALT 96 IU/L *vs* ALT 35 IU/L, $P = 0.0025$). They observed no significant differences at 12 wk in AST and ALT between the SVT20 + RFX and placebo groups (AST 48 IU/L *vs* AST 62 IU/L, $P = 0.728$ and ALT 27 IU/L *vs* ALT 35 IU/L, $P = 0.698$, respectively). There were no significant differences in alkaline phosphatase levels between the SVT40 + RFX or the SVT20 + RFX groups compared with the placebo group. The number of patients who stopped treatment because of adverse events was significantly higher in the SVT40 + RFX group [9 (56%) of 16 patients] compared with the other two groups [2 (14%) of 14 for both groups, $P = 0.017$]. In conclusion, in patients with decompensated cirrhosis, SVT40 + RFX was associated with a significant increase in adverse events requiring treatment withdrawal, specifically liver and muscle toxicity, compared with SVT20 + RFX. In future trials evaluating the role of statins in patients with decompensated cirrhosis, simvastatin 20 mg/d should be preferred to 40 mg/d. See, Safety, Reduced activities in statins metabolism and/or transport.

EFFICACY

Statins and risks of cirrhosis decompensation and death

Cirrhosis is a worldwide public health problem, together with its complications and hepatocellular carcinoma (HCC). In this regard, evidence from preclinical and clinical research of drugs such as statins showed a potentially positive effect on the natural history of cirrhosis[49].

The evaluation of these issues was performed based on large-sized national registries. These trials were derived from detailed patient databases in which the International Classification of Diseases, 9th version codes identified variables of interest. Patients treated with statins during follow-up were compared with those not treated with them, and endpoints were adjusted for the most important confounding variables. Simvastatin was the most frequent statin used in all studies. The majority of studies carried out unmatched analysis together with propensity score (PS)-matched analysis. Statins consistently reduced the risk of cirrhosis decompensation and death (Table 1).

Supported by data taken from the Veteran Affairs Clinical Case Registry, the use of statins within patients with hepatitis C virus (HCV) and compensated cirrhosis is related to a 40% or higher reduction in the risk of cirrhosis decompensation and death [50]. Huang *et al*[51] showed that patients with chronic hepatitis B who received statin had a dose-dependent reduction in the risk of cirrhosis complications. Patients with hepatitis B virus (HBV)-, HCV- and alcohol-related cirrhosis were identified from a Taiwan National Health Insurance[52]. Statins, in a dose-dependent manner, decrease the decompensation rate in both HBV- and HCV-related cirrhosis. For patients with alcohol-related cirrhosis, a decreased decompensation rate has a borderline significance. In a low number of patients with biopsy-proven cirrhosis, Kumar *et al*[53] demonstrated that statins delay cirrhosis complications and reduce mortality. For patients with alcoholic cirrhosis, the use of statins showed an association with a reduced risk of decompensation and death. This association was stronger in patients with cirrhosis than in those with non-cirrhotic controls[54]. Statins consistently reduced the risk of decompensation and death in all studies, with hazard ratios ranging from 0.29 to 0.58 and 0.46 to 0.66, respectively[55].

Statins and portal hypertension

In the first RCT on the matter, Abraldes *et al*[16] assumed that pleiotropic effects are the rational basis for statin use in patients with cirrhosis and portal hypertension, which led them to conduct a proof-of-concept study, as shown in Table 2. A multicenter RCT evaluated simvastatin or placebo administration to patients with cirrhosis and severe portal hypertension for a month. The decrease in hepatic venous pressure gradient (HVPG) by simvastatin was significantly greater than the placebo changes. In the simvastatin group, 9 out of 28 patients (32%) *vs* in the placebo group, 3 out of 27 patients (11%) reached a target hemodynamic response defined as a reduction in HVPG of at least 20% of baseline values or to less than 12 mmHg (P = 0.054). The decrease in portal pressure observed with simvastatin was not associated with changes in hepatic blood flow. Simvastatin significantly decreased the HVPG in both sets of patients: those who received NSBBs and those who did not receive them. The portal pressure-reducing effect of simvastatin was slightly higher in patients receiving NSBBs than those who were not. Finally, simvastatin administration but not placebo markedly increased indocyanine green clearance, fractional clearance and intrinsic clearance, suggesting that simvastatin increased effective liver perfusion and improved liver function. In summary, this proof-of-concept study has shown that simvastatin lowers portal pressure in patients with cirrhosis and portal hypertension, most likely through a reduction in hepatic vascular resistance without adverse effects on the systemic circulation and consistent with the theory that hemodynamic effects of simvastatin are liver-selective. Whether the patients are taking NSBBs or not, simvastatin decreases HVPG. This suggests that its effect on portal pressure is additive with the NSBBs. Besides, simvastatin improves liver function tests by enhancing effective liver perfusion and providing potential additional benefits to NSBB therapy.

The BLEPS trial is the largest RCT that assessed the effects of statins in cirrhosis. In patients with variceal bleeding, simvastatin was added to standard prophylaxis to prevent rebleeding — NSBBs and band ligation[40]. Within 10 d of bleeding, patients were randomly assigned to receive simvastatin 20 mg/d (n = 69) or placebo (n = 78) for the first 2 wk, followed by 40 mg/d or placebo for 2 years and stratified by CTP class A/B *vs* class C. The main endpoint was a composite of rebleeding or death. During a median follow-up of approximately 1 year, 30 out of 78 patients (39%) in the placebo group and 22 out of 69 patients (32%) in the simvastatin group reached the primary endpoint [hazard ratio (HR) = 0.82; 95%CI: 0.47-1.43, P = 0.420]. Nonetheless, when only death was evaluated, mortality was 22% in the placebo group compared to 9% in the simvastatin group (HR = 0.39; 95%CI: 0.15-0.99, P = 0.030). Therefore, treatment with simvastatin was associated with a 61% reduction in the relative death risk than placebo. Rebleeding, spontaneous bacterial peritonitis, and progression of liver disease were the most frequent cause of death in the placebo group (5, 3 and 3 patients, respectively), and in the simvastatin group, only one patient died by re-

Table 1 Statins, cirrhosis decompensation, hepatocellular carcinoma and mortality

Ref.	Groups, *n*	Follow up	Risk reduction
Mohanty *et al*[50], 2016	Statins 1323; Nonusers 12522	Approximately 2.5 yr for statin; Approximately 2.5 yr for nonusers	Cirrhosis decompensation and mortality
Huang *et al*[51], 2016	Statins 6543; Nonusers 6543	4.7 yr for statins; 4.6 yr for nonusers	Cirrhosis decompensation
Chang *et al*[52], 2017	Statins 1174; Nonusers 6453	Approximately 3.0 yr	Cirrhosis decompensation, HCC, and mortality
Kumar *et al*[53], 2014	Statins 81; Nonusers 162	Approximately 3.0 yr for statin; Approximately 1.5 yr for nonusers	Cirrhosis decompensation and mortality
Bang *et al*[54], 2017	Statins 794; Nonusers 4623	Approximately 4.0 yr	Cirrhosis decompensation and mortality

HCC: Hepatocellular carcinoma.

Table 2 Statins and portal hypertension

Ref.	Groups	*n*	Endpoints	Outcomes
Abraldes *et al*[16], 2009	SVT; Placebo	27; 28	Change in HVPG	HVPG decreased in SVT from 18.5 to 17.1, $P = 0.003$; no decrease in placebo
Pollo-Flores *et al*[38], 2015	SVT; Placebo	14; 20	Change in HVPG	HVPG decreased in SVT *vs* placebo: -2 *vs* 0 mmHg, $P = 0.02$
Bishnu *et al*[39], 2018	ATV + NSBBs; NSBBs	11; 12	Change in HVPG	HVPG decreased in ATV + NSBBs -4.8 *vs* NSBBs -2.6 mmHg, $P = 0.041$
Abraldes *et al*[40], 2016	SVT; Placebo	69; 78	Composite endpoint (rebleeding or death)	Non-significant decrease in risk of rebleeding or death; decrease in mortality HR = 0.39, $P = 0.030$ in SVT *vs* placebo
Wani *et al*[56], 2017	CVL; CVL + SVT	101; 35	Change in HVPG	HVPG decreased 62% in CVL and 16% in CVL + SVT (CVL non-responders)

ATV: Atorvastatin; NSBBs: Non-selective β-blockers; CVL: Carvedilol; HR: Hazard ratio; HVPG: Hepatic venous pressure gradient; SVT: Simvastatin.

bleeding, none by spontaneous bacterial peritonitis and three by a progression of liver disease. An exploratory analysis showed that mortality improvement was mainly related to decreased liver-related deaths [14 in placebo and 6 events in simvastatin; absolute risk reduction = 10.39% (95%CI: 0.26-20.51]. In the subgroup analysis, the effects of simvastatin on survival were quantitatively different in CTP class A/B patients from CTP class C patients. In CTP class A/B patients, an important outcome was the significant decrease in mortality with simvastatin, which was not observed in CTP class C patients (HR = 0.16; 95%CI: 0.05-0.27, $P = 0.006$), as shown in Table 2. The other major secondary outcome was rebleeding. In the placebo group, 22 patients developed rebleeding (28%) and 17 patients in the simvastatin group (25%). The rebleeding rate was not significantly decreased by the addition of simvastatin to standard therapy. Neither difference was observed in the rate of other secondary outcomes, ascites, spontaneous bacterial peritonitis, hepatorenal syndrome, portal vein thrombosis and need for rescue shunting, need for transfusion, liver transplantation and Model for End-stage Liver Disease (MELD) score between the two groups during the study period. The BLEPS trial demonstrated that the effect of adding simvastatin to the standard of care in patients who recover from an acute variceal bleeding episode improves survival without reducing the rate of other complications of cirrhosis.

Shortly after, two RCTs on small groups of patients reported the effects of statins on portal hypertension. Most of the patients, CTP class A and class B, had compensated cirrhosis, and very few were CTP class C. In one of these trials, simvastatin (at 40 mg/d for 3 mo) was evaluated *vs* placebo, whereas for the other trial, atorvastatin (20 mg/d for 1 mo) plus propranolol was tested *vs* propranolol alone on portal pressure estimated by HVPG[38,39] (Table 2).

In the first place, Pollo-Flores *et al*[38] showed that simvastatin therapy was associated with a decrease in HVPG, whereas no change was noted in the placebo group. Likewise, in 55% of patients treated with simvastatin, a clinically significant reduction in portal pressure was achieved because the decrease in HVPG was greater

than 20% *vs* no decrease in HVPG for patients with placebo (P = 0.030). Furthermore, they observed a slight improvement in liver function by CTP score, at baseline 6.6 *vs* at the end of the trial 6.2, P = 0.080. They concluded that simvastatin lowers portal pressure and may improve liver function.

Secondly, Bishnu *et al*[39] demonstrated that atorvastatin plus propranolol *vs* propranolol alone was associated with a greater decrease of HVPG mean. Likewise, the combined treatment compared with propranolol alone achieved more frequently a clinically significant reduction in portal HVPG (91% *vs* 50%, P = 0.069). However, they did not observe differences between the groups in cirrhosis complications and death after a 1 year follow-up. They concluded that the addition of atorvastatin to propranolol leads to a more significant reduction of portal pressure in patients with cirrhosis, likely through a reduction in hepatic vascular resistance.

In 2017, Wani *et al*[56] carried out a sequential trial in carvedilol non-responders to analyze if adding simvastatin could be a medical rescue therapy in these patients. One hundred and two consecutive patients with cirrhosis and significant portal hypertension were included. Forty-three were CTP class A, 32 were CTP class B and 27 were CTP class C. HVPG was measured at the baseline and after 3 mo of carvedilol treatment. Sixty-four patients (63%) responded to carvedilol. The other thirty-eight were classified as carvedilol non-responders. In these patients, simvastatin 20 mg/d was added for 2 wk, and after the adverse events were evaluated, 3 patients were discontinued. Thirty-five continued carvedilol and simvastatin 40 mg/d for up to 1 mo, and a new measurement of HVPG was performed. There were 16 responders (42%) and 19 non-responders. Thus, the overall carvedilol response was 79% (63% with carvedilol alone and 16% with carvedilol plus simvastatin), as shown in Table 2. In conclusion, this is the first time it has been clearly shown that a sequential treatment is an excellent strategy in the medical therapy of portal hypertension.

Statins and risk of infections

Infections are common in cirrhotic patients, and their presence causes a four-fold increase in mortality[57]. Motzkus-Feagans *et al*[58] assessed the consequence of including statins on the occurrence of infections. This trial was carried over 19379 compensated cirrhosis patients from the United States Veterans Health Administration database. Statins were administered to 2468 patients, simvastatin being the most common one. Infection was diagnosed in 200 statin users and 2153 nonusers during follow-up. The most frequent infections were pneumonia and skin infections. Statin treatment was associated with a reduction of infection rate and of mortality rate in the whole cohort (adjusted HR = 0.42; 95%CI: 0.36-0.48), and the PS-matched sample included 503 statin users and 1760 statin nonusers (adjusted HR = 0.67; 95%CI: 0.47-0.95). They concluded that statin use might potentially reduce the morbidity and mortality by infections in patients with cirrhosis.

Statins and risk of HCC

The World Health Organization (globacan.iarc.fr) estimated that HCC globally speaking is now the fifth most common type of cancer and the third cause of cancer-related mortality. Back in 2012, there were approximately 782000 cases, of which 83% were from the underdeveloped regions of the world[59]. Pre-existing cirrhosis was present in more than 80% of participants diagnosed with HCC[60].

The Electronically Retrieved Cohort of HCV Infected Veterans for the first time has shown that statin addition to pegylated interferon-based therapy for chronic HCV infection, decreases the incidence of HCC in those who received statin therapy compared to those who did not. Statins remained significantly associated with a lower risk of HCC incidence (HR = 0.51; 95%CI: 0.34-0.76)[61]. In an extension of their previous study, the authors showed that the decrease in HCC was dose-dependent. For subjects with 90-180 cumulative defined daily dose and cumulative defined daily dose > 180, adjusted HRs of incident HCC were 0.48 (95%CI: 0.27-0.88, P = 0.02) and 0.51 (95%CI: 0.36-0.72, P = 0.0001), respectively. Increasing statin dose was also associated with a significant delay in the time taken for the development of HCC. In two sensitivity analyses, it was shown that the incidence of HCC was independent of statin antifibrotic effect and sustained virological response[62].

Hsiang *et al*[63] ran a hospital-based population trial on HBV patients taken from the Hospital Authority database in Hong Kong. They defined statin use by landmark analysis to abrogate "immortal time bias" and PS to minimize baseline confounders and "indication bias." The 2-year landmark analysis entered 73499 patients with a crude HCC incidence of 1.75 per 100 patient-years. After landmark analysis and PS weighting of baseline covariates, statin users had a 32% risk reduction of HCC than nonusers. In subgroup analysis, a reduction of 59% in the HCC risk was associated

with concomitant statin and nucleos(t)ide analogues use compared to nucleos(t)ide analogues use alone. In conclusion, after adjustment for confounders and biases in this HBV cohort, statin use was associated with reduced HCC risk by 32%. The authors were able to see the additive HCC chemopreventive effect with the concomitant use of nucleos(t)ide analogues and statins. In 2018, Kim *et al*[64] investigated the risk of HCC after statin use in the whole population and patients at high risk for pre-existing diabetes or cirrhosis. A nationwide, nested case-control study was conducted with data from the National Health Insurance Service-Physical Health Examination Cohort 2002-2013 in the Republic of Korea. Individuals diagnosed with HCC, over 514000 participants, were matched with controls based on the follow-up, their sex and their age. Statin use was associated with a reduced risk of HCC development [adjusted odds ratio (aOR) = 0.44] than nonusers. The reduction in patients at risk was more significant in the presence of diabetes (aOR = 0.28) than in the absence of it (aOR = 0.53) and the presence of cirrhosis (aOR = 0.39) than in the respective absence (aOR = 0.42). In conclusion, statin use may have a beneficial inhibitory effect on HCC development, particularly in patients at high risk for pre-existing diabetes or cirrhosis.

Statins have also been tried as concomitant therapy in patients with confirmed HCC. Two RCTs evaluated the role of statins in the treatment of advanced HCC. Japanese authors randomized 83 patients with non-resectable HCC undergoing transarterial chemoembolization (TACE) into pravastatin 40 mg/d group and control group. The mean survival rate resulted significantly longer in the pravastatin plus TACE group *vs* control group, over 18 mo *vs* 9 mo, respectively (relative risk = 0.35; 95%CI: 0.17-0.61, P = 0.005)[65]. These results were confirmed in a similarly designed German RCT that included 131 patients. Survival in the pravastatin plus TACE group was 20.9 mo compared to TACE alone group 12.0 mo, P = 0.003[66].

OUR EXPERIENCE

A safety prospective, open, uncontrolled phase IIa trial was recently published online ahead of print about chronic simvastatin treatment in patients with decompensated cirrhosis. This trial included 30 patients, CTP class A (n = 6), B (n = 22) and C (n = 2). The patients received standard treatment plus simvastatin 20 mg/d for 2 wk and after that simvastatin 40 mg/d up to 1 year[67].

Muscle injury was assessed according to the National Lipid Association Safety Expert Panel of the United States[36]. In total, 11 out of 30 patients (36%) developed muscle injury, of which 7 were classified as myalgia (23.4%) and the remaining 4 myonecrosis (13.3%). All of them were related to simvastatin. On the other hand, a significantly greater baseline MELD score was observed within the group with muscle injury (14.0 ± 3.6) than the group without muscle injury (11.4 ± 2.8) (P = 0.035). The receiver operating characteristic analysis revealed a cutoff value > 12 for baseline MELD score to differentiate patients with muscle injury from those without muscle injury. Moreover, the only two CTP class C patients included in the study developed myonecrosis.

No patient developed drug-induced liver injury according to the criteria proposed by Aithal *et al*[68]. One patient developed diabetes mellitus (3.3%). This rate is more significant than for the first time reported in the JUPITER trial (0.6%)[69]. Gastrointestinal symptoms were recorded in 53.3% of patients. The rate of each of them was from 7% to 40%. This figure is more significant than reported with simvastatin in subjects without liver disease for all digestive symptoms (10% and each one of them from 0.7% to 2.5%)[17]. Simvastatin dosage was reduced exclusively by myalgia and transiently interrupted due to myonecrosis; thus, muscle injury was considered the only clinically significant adverse event.

In conclusion, chronic treatment with simvastatin 40 mg/d in patients with decompensated cirrhosis up to 1 year was associated with several adverse events, although no liver injury was registered. Moreover, muscle injury was the only clinically significant adverse event, which appears to be related to the simvastatin dosage and the degree of cirrhosis severity. Consequently, simvastatin 40 mg/d should not be prescribed in patients with cirrhosis MELD score > 12 because of a high rate of adverse muscle events and CTP class C patients due to potential severe muscle injury.

META-ANALYSIS AND EDITORIAL

A recent systematic review and meta-analysis discussed the impact of statins on fibrosis progression, hepatic decompensation and death in patients with chronic liver disease[70]. Ten trials spanning over 259453 patients were evaluated; most of the aforementioned studies were discussed in this review. Subjects receiving statins *vs* statin nonusers were associated with a reduction of all the previous endpoints mentioned. However, the quality of the evidence for these results was low or very low because nine out of ten studies were observational.

An editorial about this article highlights that the quality of these outcomes arises from the weakness of observational studies, selection or channelings bias, residual confounding, heterogeneity in the methods used to estimate fibrosis progression, the underlying population, lack of granularity regarding specific statin use, dose and the absence of information on adverse events of statins[71].

Finally, the authors proposed if the time has arrived to consider approving the use of statins in patients with liver disease to prevent liver-related morbidity and mortality. There are now experimental studies supporting the biological plausibility of hepatic protection, observational evidence of a beneficial effect and a limited number of RCTs demonstrating benefits. However, many unknowns remain, including the optimal statin to select and its dosing, particularly in patients with impaired liver function, and the risk of severe non-hepatic adverse events, such as rhabdomyolysis [71]. After Kamal *et al*[70]'s study in 2017, only the LIVERHOPE-SAFETY trial[42] and our safety study[67] provided some security data, as we will refer to in the Discussion and Conclusion. Nevertheless, the study by Kamal *et al*[70] encourages and supports the need for well-designed clinical trials to assess the benefits and safety of statins in this population.

CONCLUSION

In patients with cirrhosis, the most significant adverse event is statin-related myopathy, and it may be associated with high serum statin concentrations in the setting of severely impaired liver function. According to the LIVERHOPE-SAFETY trial findings, patients with decompensated cirrhosis should be treated with a lower dose of simvastatin, maybe 20 mg/d rather than 40 mg/d dose. For this reason, statins could be unsafe in CTP class C patients. Likewise, and based on the safety trial, we advise that a simvastatin dosage of 40 mg/d should not be prescribed in patients with cirrhosis MELD score > 12 and/or CTP class C patients due to potential severe muscle injury. In observational studies over large populations of cirrhosis patients, statins were associated with a reduced risk of hepatic decompensation, HCC development and death. However, the quality of the evidence of these studies is considered low or very low. The few RCTs in patients with cirrhosis and portal hypertension showed statins lower portal pressure, likely reducing hepatic resistance. The largest RCT demonstrated that the addition of simvastatin to standard therapy improved survival but did not prevent variceal rebleeding. For these reasons, before endorsing statins in patients with CTP class A/B cirrhosis to prevent liver-related morbidity and mortality, further RCTs should be performed over a larger number of patients with hard clinical endpoints using different statins and dosage[72,73].

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MINIREVIEWS

Genetic variant of cyclooxygenase-2 in gastric cancer: More inflammation and susceptibility

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Abstract

Gastric cancer accounts for the majority cancer-related deaths worldwide. Although various methods have considerably improved the screening, diagnosis, and treatment of gastric cancer, its incidence is still high in Asia, and the 5-year survival rate of advanced gastric cancer patients is only 10%-20%. Therefore, more effective drugs and better screening strategies are needed for reducing the incidence and mortality of gastric cancer. Cyclooxygenase-2 (COX-2) is considered to be the key inducible enzyme in prostaglandins (PGs) synthesis, which is involved in multiple pathways in the inflammatory response. For example, inflammatory cytokines stimulate innate immune responses *via* Toll-like receptors and nuclear factor-kappa B to induce COX-2/PGE2 pathway. In these processes, the production of an inflammatory microenvironment promotes the occurrence of gastric cancer. Epidemiological studies have also indicated that non-steroidal anti-inflammatory drugs can reduce the risk of malignant tumors of the digestive system by blocking the effect of COX-2. However, clinical use of COX-2 inhibitors to prevent or treat gastric cancer may be limited because of potential side effects, especially in the cardiovascular system. Given these side effects and low

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treatment efficacy, new therapeutic approaches and early screening strategies are urgently needed. Some studies have shown that genetic variation in *COX-2* also play an important role in carcinogenesis. However, the genetic variation analysis in these studies is incomplete and isolated, pointing out only a few single nucleotide polymorphisms (SNPs) and the risk of gastric cancer, and no comprehensive study covering the whole gene region has been carried out. In addition, copy number variation (CNV) is not mentioned. In this review, we summarize the SNPs in the whole *COX-2* gene sequence, including exons, introns, and both the 5′ and 3′ untranslated regions. Results suggest that *COX-2* does not increase its expression through the CNV and the SNPs in *COX-2* may serve as the potential marker to establish risk stratification in the general population. This review synthesizes emerging insights of COX-2 as a biomarker in multiple studies, summarizes the association between whole *COX-2* sequence variation and susceptibility to gastric cancer, and discusses the future prospect of therapeutic intervention, which will be helpful for early screening and further research to find new approaches to gastric cancer treatment.

Key Words: Cyclooxygenase-2; Inflammation; Genetic variant; Gastric cancer; Prostaglandin E2

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Core Tip: Cyclooxygenase-2 (COX-2) is considered to be the key inducible enzyme in prostaglandins synthesis, and non-steroidal anti-inflammatory drugs can reduce the risk of malignant tumors of the digestive system by blocking the effect of COX-2. However, COX-2 inhibitors to prevent or treat gastric cancer may be limited because of their cardiovascular side effects. This review will be helpful for early screening and further research to find new approaches to gastric cancer treatment by summarizing the association between whole *COX-2* sequence variation and susceptibility to gastric cancer and synthesizing the new progress in understanding the role of COX-2 in gastric carcinogenesis.

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INTRODUCTION

Gastric cancer is the fifth most commonly diagnosed cancer and the third leading cause of cancer-related deaths worldwide. The incidence of gastric cancer remains high in Eastern Asian despite its global decrease in the last few years[1,2]. Approximately 75% of patients with gastric cancer are diagnosed at advanced stage and the median survival is 7-10 mo[3]. Therefore, individualized prevention and early detection and treatment are of clinical significance in improving the survival time and reducing the mortality of gastric cancer.

Environmental factors including smoking, drinking, and *Helicobacter pylori* (*H. pylori*) infection and genetic alterations such as susceptible genetic variants and epigenetic alterations have been associated with gastric carcinogenesis[4,5]. Cyclooxygenase-2 (COX-2) has been extensively studied in carcinogenesis, and its participation in chronic inflammation and various infections (such as *H. pylori* infection and chronic viral hepatitis) significantly increases the risk of cancer[6,7]. In this review, we will summarize the association between whole *COX-2* sequence variation and susceptibility to gastric cancer. We will also discuss the crucial role of *COX-2* in the occurrence of gastric cancer and its mechanisms.

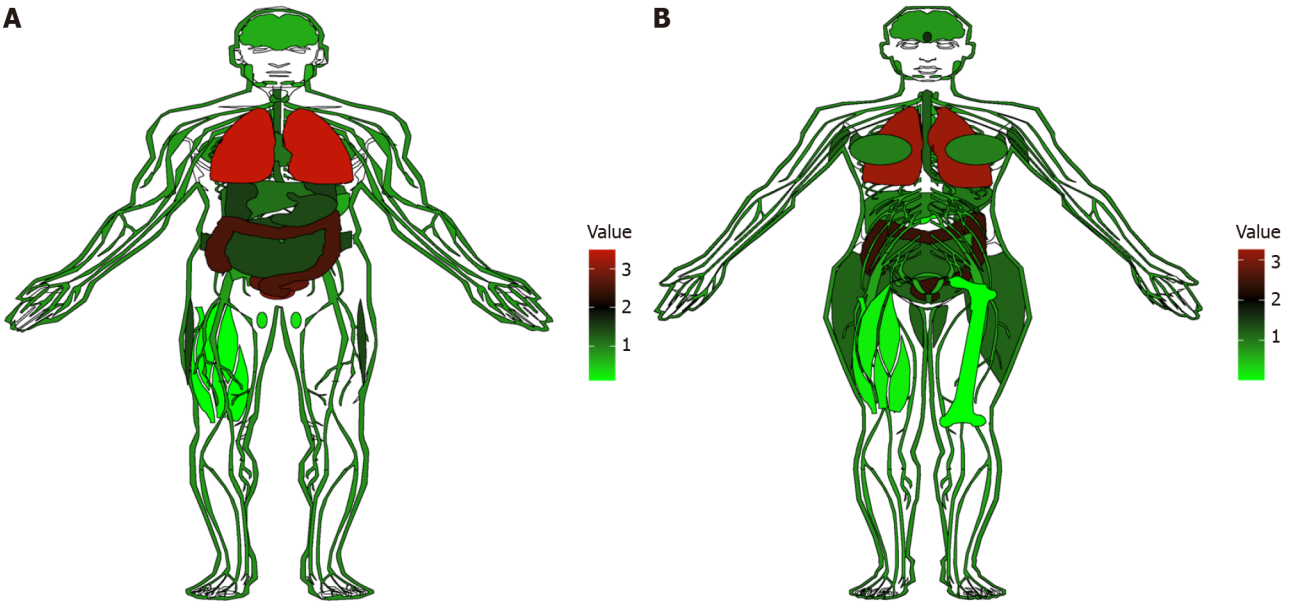


Figure 1 Visualization of cyclooxygenase-2 expression in normal tissues from the genotypic tissue expression database. The red parts indicate the organs with more cyclooxygenase-2 distribution, while the green parts indicate less distribution. A: Distribution of expression in males; B: Distribution of expression in females.

MOLECULAR CHARACTERISTICS OF COX-2

COX-2 is known as the key inducible enzyme in prostaglandins (PGs) synthesis, and the *COX-2* gene is located at chromosome 1q25.2-25.3 and composed of 9 introns and 10 exons[8]. The 5′ region of the *COX-2* gene has binding sites for several activated transcriptional factors, such as nuclear factor-kappa B (NF-κB), stimulatory protein 1 (SP1), activator protein-2 (AP-2), and transforming growth factor. In order to explore the expression of *COX-2* in normal tissues, the expression data of *COX-2* were downloaded from the genotypic tissue expression (GTEx) database (https://xenabrowser.net/datapages/) and the distribution of *COX-2* expression in different tissues was visualized by plotting an anatomical map with R-3.5.3 software. Detailed data are shown in Supplementary material 1. Previous studies have shown that *COX-2* has negative expression in normal tissues and organs under physiological conditions, though it is constitutively expressed in the brain and kidney. We also found that *COX-2* gene was rarely expressed in normal tissues (including the stomach), but distributed more in the colon and lungs, both in males and females (Figure 1). However, its expression is increased dramatically in response to certain inflammatory stimuli such as cytokines, oncogenes, and tumor inducers[9]. COX-2 have been shown to play crucial roles in tumorigenesis[10]. The COX-2/PGE2 pathway activates macrophage infiltration and further induces cytokine signaling to activate the transcription factors NF-κB and signal transducer and activator of transcription 3 (Stat3)[11,12], which can change the tumor microenvironment and affect the occurrence of cancer.

GENETIC VARIANTS OF COX-2 IN TUMORIGENESIS

COX-2 has been implicated in the etiology of cancer and its expression has been confirmed to be increased in gastric cancer. Genetic variants may lead to an increase in expression and change in the function of *COX-2*, which may affect the occurrence of cancer. Studies have suggested that *COX-2* single nucleotide polymorphisms (SNPs) may affect the gastric tumorigenesis. However, these studies only focused on a few or particular region SNPs, and lacked an overall description of the whole sequence variation of *COX-2*. In this review, we summarize the SNPs in the whole *COX-2* gene sequence, including exons, introns, and both the 5′ and 3′ untranslated regions (UTR). In addition, we also analyze the copy number variation (CNV) information of *COX-2* in gastric cancer.

CNV of COX-2 in gastric cancer

The SNPs of *COX-2* have been widely studied, but its CNV was rarely mentioned. We downloaded the copy number data of the *COX-2* gene in gastric cancer from The Cancer Genome Atlas (TCGA) database (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga), and then visualized the data with R-3.5.3 software (detailed data in Supplementary material 2). The genes displayed are all genes with CNV, but no CNV of the *COX-2* gene was found.

Association between COX-2 SNPs and gastric cancer

The SNPs of *COX-2* may have a functional effect on *COX-2* transcription and cause *COX-2* overexpression to change the response to various inflammatory stimuli. However, only a single locus of SNP can explain the occurrence of cancer very little, which is not enough to fully demonstrate the association between *COX-2* SNPs and gastric cancer. We combined data from the TCGA (https://portal.gdc.cancer.gov/repository; downloaded data in Supplementary material 3) and Ensembl (http://grch37.ensembl.org/Homo_sapiens/Tools/VcftoPed?db=core) using Haploview 4.2 software to screen SNPs. The criteria for screening SNPs were minor allele frequency ≥ 0.05 and pairwise $r^2 < 0.8$. All obtained SNPs are shown in Figure 2. At the same time, we retrieved the SNPs that have been studied. The results showed that 14 SNPs were associated with cancer in the whole sequence of *COX-2*, including 9 SNPs associated with gastric cancer (Table 1). At present, five *COX-2* polymorphisms have been extensively studied, including rs5275 and rs689470T>C that are located in the 3′ UTR, as well as rs689466G>A and rs20417G>C mutations that are located in the promoter region with multiple enhancers and transcriptional regulatory elements. SNPs in the *COX-2* promoter region may change the activity of the promoter and C-reactive protein (CRP), which may be related to acute or chronic inflammation[13]. Although SNPs may have functional effects, there are still a large number of functional features of SNPs that have not been discovered, and their mechanism needs to be further studied. Meanwhile, risk estimates of previous studies have been inconsistent. Therefore, we made a summary and pooled analysis of the extracted data. The results showed that rs689466G>A, rs20417G>C, and rs3218625G>A in the promoter region conferred a higher risk of gastric cancer [A *vs* G: odds ratio (OR) = 1.19, 95% confidence interval (CI): 1.10-1.29; C *vs* G: OR = 1.26, 95%CI: 1.12-1.41; and A *vs* G: OR = 1.62; 95%CI: 1.02-2.56]. Similarly, rs5275T>C and rs689470T>C in the 3′UTR were significantly associated with gastric cancer (C *vs* T: OR = 1.14, 95%CI: 1.01-1.29 and TC *vs* TT: OR = 7.49; 95%CI: 1.21-46.2). As to the rs2066826 G>A polymorphism, a significant association was detected in pancreatic cancer (A *vs* G: OR = 1.60, 95%CI: 1.06-2.40, $P = 0.026$). However, rs5279 T>C in the exon region and rs4648298A>G in the 3′ UTR may reduce the risk of gastric and colorectal cancers (TC *vs* TT: OR = 0.24, 95%CI: 0.08-0.73 and G *vs* A: OR = 0.24; 95%CI: 0.10-0.56).

In our previous study of 296 gastric cancer patients and 319 control family members in the Chinese Han population, an increased risk was observed in individuals with the *COX-2* rs689466AA genotype (OR = 2.03; 95%CI: 1.27-3.22), and the association decreased as the degree of relationship decreased[14]. Recently, we further performed genotyping in 660 gastric cancer cases form the First Affiliated Hospital of Zhengzhou University from 2013 to 2015 and 660 control individuals from a community-based cardiovascular diseases survey in the same time. Our results found that individuals with rs20417 GC genotype were more likely to develop gastric cancer (OR = 1.54, 95%CI: 1.08-2.19). Meanwhile, Zhang *et al*[15] found that rs689466 G>A enhanced the transcriptional activity and thus increased the expression of *COX-2* by creating a c-MYB binding site.

These results suggest that the SNPs of the *COX-2* gene plays an important role in the carcinogenesis of gastric cancer, especially the variation in the promoter region which may have functional consequences. In addition, SNPs in the promoter region could enhance *COX-2* gene transcription, affect the stability of mRNA, regulate the inflammatory response, and consequently lead to individual variation in susceptibility to gastric cancer[16,17]. Our study provides a basis for more thoroughly exploring the exact function of *COX-2* in the occurrence of gastric cancer. Further functional studies will be considered and be elaborated in another study.

INFLAMMATORY MECHANISM OF COX-2 IN GASTRIC CANCER

COX-2 overexpression has been found in a variety of cancers, including gastric cancer [18,19]. A large number of studies have shown that many factors (such as *H. pylori*

Table 1 Summary of single nucleotide polymorphisms in whole region of cyclooxygenase-2 and their association with risk of cancer

SNP ID (rs number)	Chromosome location (GRCh38)	Cancer type	Model	Region	Effect	MAF	OR (95%CI)	*P* value	Ref.
rs689465A>G	1:186681714	Gastric	G/A	Promoter	Significant interaction with *H. pylori* infection	0.143	0.84 (0.57-1.24)	0.381	Zhang *et al*[55]
rs689466G>A	1:186681619	Gastric	A/G	Promoter	Increases transcriptional activity by creating a c-MYB binding site	0.176	1.19 (1.10-1.29)	0.002[a]	Li *et al*[14], Piranda *et al*[16], Zhang *et al*[55], Lopes *et al*[56], Liu *et al*[57], Shin *et al*[58], Guo *et al*[59], Zamudio *et al*[60], and Luo *et al*[61]
rs20417G>C	1:186681189	Gastric	C/G	Promoter	Reduces promoter activity by creating a binding site for NPM and P-NPM	0.109	1.26 (1.12-1.41)	< 0.001[a]	Li *et al*[14], Piranda *et al*[16], Liu *et al*[17], Zhang *et al*[55], Shin *et al*[58], Sitarz *et al*[62], Saxena *et al*[63], Hou *et al*[64], Zhang *et al*[65], Campanholo *et al*[66], He *et al*[67], and Di Marco *et al*[68]
rs3218625G>A	1:186674409	Gastric	A/G	Promoter	Enhances activity of *COX-2 in vitro* by causing the transition of Gly to Aly	< 0.001	1.62 (1.02-2.56)	0.039[a]	Zhang *et al*[55] and Zhang *et al*[65]
rs5277G>C	1:186679065	Gastric	GC/GG	Exon	-	0.108	0.42 (0.13-1.28)	0.127	Hussain *et al*[69]
rs5278T>C	1:186676537	Gastric	TC/TT	Exon	-	0.021	2.27 (0.53-9.69)	0.270	Hussain *et al*[69]
rs5279T>C	1:186675946	Gastric	TC/TT	Exon	-	0.015	0.24 (0.08-0.73)	0.012[a]	Hussain *et al*[69]
rs2745557G>A	1:186680089	Pancreatic	A/G	Intron	-	0.164	0.94 (0.64-1.39)	0.764	Özhan *et al*[70]
rs2066826G>A	1:186676795	Pancreatic	A/G	Intron	-	0.188	1.60 (1.06-2.40)	0.026[a]	Özhan *et al*[70]
rs4648262G>T	1:186679617	Pancreatic	T/G	Intron	-	< 0.001	0.62 (0.22-1.73)	0.364	Özhan *et al*[70]
rs4648298A>G	1:186672550	Colorectal	G/A	3′-UTR	Creates a longer and possibly more stable species of mRNA	0.021	0.44 (0.25-0.75)	0.003[a]	Gholami *et al*[71], Mosallaei *et al*[72], and Cox *et al*[73]
rs5275T>C	1:186673926	Gastric	C/T	3′-UTR	Stabilizes mRNA of *COX-2* by potential miRNA-binding sites	0.351	1.14 (1.01-1.29)	0.030[a]	Piranda *et al*[16], Li *et al*[74], and Furuya *et al*[75]
rs689470T>C	1:186671926	Gastric	TC/TT	3′-UTR	Degradation of the mRNA	0.039	7.49 (1.21-46.20)	0.030[a]	Hussain *et al*[69] and Hu *et al*[76]
rs2206593A>G	1:186673297	Breast	G/A	3′-UTR	-	0.060	0.92 (0.84-1.91)	0.088	Li *et al*[77]

[a]$P < 0.05$. SNP: Single nucleotide polymorphism; NPM: Nuceophosmin; P-NPM: Phosphorylated NPM; OR: Odds ratio; CI: Confidence interval; COX-2: Cyclooxygenase-2.

infection, NF-κB activation, K-ras expression, and the dysregulation of some trans-acting regulatory factors) can lead to overexpression of COX-2 and more inflammation in neoplasia[20-23].

H. pylori infection and COX-2 expression

It is generally accepted that *H. pylori* infection is an important risk factor for gastric cancer and *H. pylori* has been classified as a class I carcinogen. *H. pylori* infection may

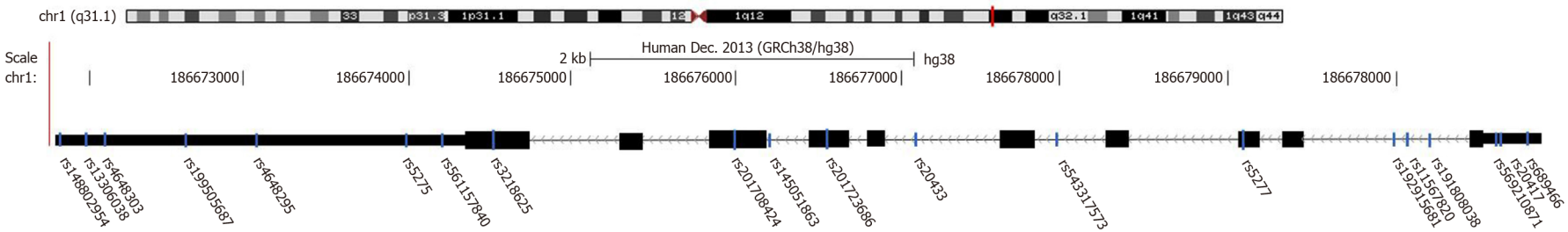


Figure 2 General location of cyclooxygenase-2 single nucleotide polymorphisms searched by bioinformatics (based on The Cancer Genome Atlas, NCBI, and UCSC).

trigger various inflammatory pathways to increase cancer risk. A study has shown that 24 h after *H. pylori* infection of the MKN 28 cell line, the level of *COX-2* mRNA transcription and PGE2 expression increased 5-fold and 3-fold, respectively[24]. However, the exact molecular mechanisms underlying the increased expression of COX-2 in gastric cancer patients with *H. pylori* infection remains unclear.

A study found that *H. pylori* infection stimulates Toll-like receptors (TLRs), to activate innate immunity and the COX-2/PGE2 pathway, which induces "infection-associated inflammation" [such as CXCL1, 2, and 5, CCL3 and 4, interleukin (IL)-11, IL-23, and tumor necrosis factor alpha (TNF-α)], to generate an inflammatory microenvironment and further lead to gastric tumorigenesis[25,26]. Another study using AGS gastric cancer cells showed that *H. pylori* (patient isolate) promotes *COX-2* transcription, which may be due to the activation of mitogen-activated protein kinase (MAPK) pathways (ERK1/2, p38, and JNK) and the activation of cAMP response element (CRE) and AP-1 on the *COX-2* promoter by TLR2/TRL9[27]. Jüttner *et al*[28] found that the binding of upstream stimulatory factor 1/2 (USF1 and 2) to the CRE/Ebox site of the *COX-2* promoter promotes the upregulation of *COX-2* after *H. pylori* infection. Another study demonstrated that *H. pylori* infection may lead to the phosphorylation of p38 MAPK and its downstream activating transcription factor 2 (ATF-2), which affects the expression of COX-2[29]. Some studies have pointed out that the expression of COX-2 is induced by NF-κB, which is recognized and activated by c-Src or TLR2/TLR9 and MAPK kinase kinase 14 (MAP3K = NIK)[30]. In addition, *H. pylori* infection promoted the secretion of gastrin, which extended the half-life of *COX-2* mRNA and increased the expression of COX-2[31]. Semple *et al*[32] reported that gastrin upregulates the expression of COX-2 by CCK-2R-mediated JAK2/Stat3 and subsequent PI3K/Akt activation in gastric cancer cell lines. Meanwhile, *H. pylori* infection may also activate NF-κB, which can induce COX-2 expression[33]. Moreover, another study showed that eradication of *H. pylori* infection significantly reduced COX-2 expression[34].

Table 2 Application of cyclooxygenase-2 inhibitors in cancer

Drug	Cancer type	Effect(s)	Mechanism	Ref.
Celecoxib	Gastric	Inhibits tumor growth	Increases CD206 and activates macrophages	Thiel *et al*[46]
Aspirin	Gastric	Induces apoptosis; inhibits proliferation; inhibits angiogenesis	Acetylates the active site of COX-2;inhibits PG synthesis; activates caspase-8/Bid and caspase-3	Liu *et al*[57], and Niikura *et al*[78]
Oxadiazole 10c	Colon	Increases antitumor activity; increases sensitivity	Docked into the COX-2 bind site	El-Husseiny *et al*[79]
Celecoxib and platinum	Gastric	Prolong overall survival and progression-free survival	-	Guo *et al*[80]
Celecoxib and rapamycin	Gastric	Increase sensitivity	Inhibit PI3K/AKT pathway	Cao *et al*[81]
Celecoxib and FOLFOX4	Gastric	Inhibit angiogenesis	Down-regulate VEGF level	Tołoczko-Iwaniuk *et al*[82]
Celecoxib and erlotinib	Colorectal	Reduce angiogenesis; inhibit formation; inhibit expansion	Inhibit EGFR signaling	Roberts *et al*[83]
Celecoxib and Curcumin	Hepatocellular	Inhibit angiogenesis; inhibit proliferation; induce apoptosis	Inhibit Akt/NF-κB/PGE2/ROS pathway; inhibit COX-2/PGE2 pathway	Abdallah *et al*[84]
Sorafenib and meloxicam	Hepatocellular	Inhibit tumor cell growth; inhibit proliferation; enhance apoptosis	Activate endoplasmic reticulum stress; enhance the cytotoxicity	Zhong *et al*[85]
Ferrocene derivatives	Breast/cervical	Suppress tumor growth; increase antiproliferative activity; induce apoptosis	Increase the levels of cytotoxicity and reactive oxygen species; reduce the level of PGE2	Ren *et al*[86], and Farzaneh *et al*[87]

COX-2: Cyclooxygenase-2; PGs: Prostaglandins; PI3K: Phosphatidylinositol 3 kinase; Akt (PKB): Protein kinase B; VEGF: Vascular endothelial growth factor; PGE2: Prostaglandin E2; ROS (MDA): Reactive oxygen species measured as malondialdehyde.

Noteworthy, COX-2 is overexpressed not only in *H. pylori* positive gastritis and gastric cancer, but also in precancerous lesions such as intestinal metaplasia and atrophic gastritis, suggesting that COX-2 plays a key role in the early gastric carcinogenesis[35,36]. These may be associated with individual genetic susceptibility, especially inflammatory genes, such as *COX-2*, *IL-1b*, and *TNF-α* gene polymorphisms in our previous reports[14,37].

Inflammatory pathway of COX-2

COX-2 is regulated by multiple pathways in gastric cancer cell lines. The pathway of COX-2/PGE2 has been shown to play crucial roles in tumorigenesis by triggering the production of an inflammatory microenvironment[10,38,39]. However, the exact tumor-promoting mechanism of PGE2 remains unclear. It has been reported that TLR signaling through the adaptor molecule MyD88 induces the COX-2/PGE2 pathway to promote the occurrence of gastritis and gastric cancer[26]. Meanwhile, the expression of COX-2 was significantly reduced when NF-κB signal was blocked by chondroitin sulfate[40]. Some inflammatory cytokines, such as IL-6, IL-8, and TNF-α, can activate NF-κB to induce overexpression of COX-2[41].

It has also been reported that the expression of K-ras and the activation of matrix metalloproteinase-2 (MMP-2) and MMP-9 are significantly related to the increased expression of COX-2[42]. They may jointly promote the occurrence of gastric cancer, but the mechanism is not clear.

Recent studies suggest that the cooperation of the COX-2 /PGE2 pathways and TLR/MyD88 signaling through NF-κB activation is crucial in tumorigenesis[26]. Some genetic studies have shown that the activation of carcinogenic Wnt is related to the occurrence of gastric tumors induced by COX-2[10,38]. In the TCGA database, the Wnt signal and the NF-κ B and COX-2 inflammatory pathways were observed to be activated simultaneously in intestinal gastric cancer[26]. The adenomatous polyposis coli (APC) regulates the expression of COX-2 through a β-catenin-independent mechanism[43]. Inducible nitric oxide synthase (iNOS) can increase the activity of COX-2 to upregulate the production of PGs[44].

These results suggest that COX-2 promotes the occurrence of cancer through induction of various inflammatory pathway signaling and generation of an inflammatory microenvironment (Figure 3).

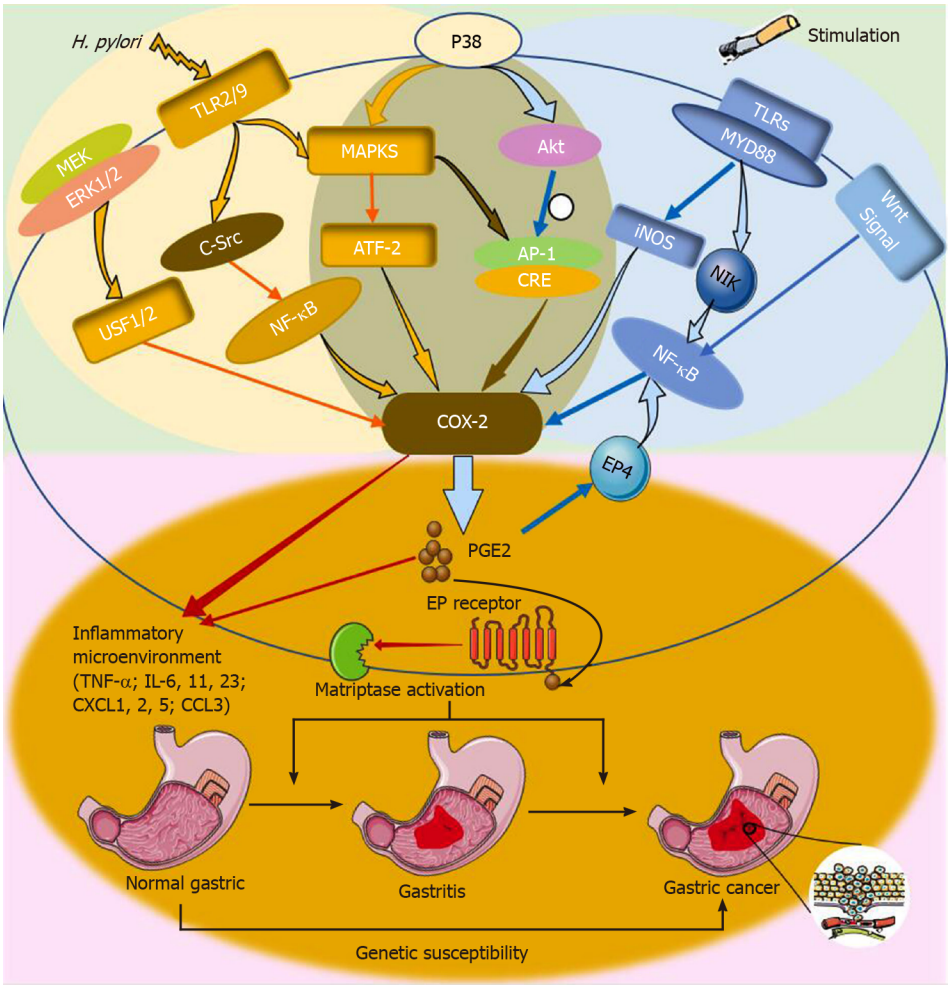


Figure 3 Schematic representation of interactions in regulation of cyclooxygenase-2 to generate an inflammatory microenvironment that has been described in the review. The active pathway of *Helicobacter pylori* infection is shown on the right. TLR: Toll-like receptor; NF-κB: Nuclear factor κB; PGE2: Prostaglandin E2; EP4: Prostaglandin E receptor subtype 4; C-Src: Cellular src; Akt: Protein kinase B; AP-1: Activator protein 1; MEK1: MAP kinase kinase 1; USF: Upstream stimulatory factor; NIK: Mitogen-activated protein kinase kinase kinase 14; Inos: Inducible nitric oxide synthase; MAPK: Mitogen-activated protein kinase; ATF-2: Activating transcription factor 2; TNF-α: tumor necrosis factor-α; IL: Interleukin; CXCL: Chemokine (CXC motif) ligand; CCL: Chemokine (C-C motif) ligand; COX-2: Cyclooxygenase-2.

ROLE OF COX-2 IN CANCER PREVENTIONS AND THERAPEUTICS

Epidemiological studies have indicated that the application of COX-2 inhibitors can reduce the inflammatory response and suppresses gastrointestinal cancerization. It may be an effective and crucial target to treat patients with atrophic gastritis and reduce the risk of *H. pylori*-related gastric cancer[22,45]. The use of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin can reduce the risk of malignant tumors of the digestive system by blocking the effect of COX-2[46].

NSAIDs can reduce the number and size of colorectal carcinomas in patients with familial adenomatous polyposis. Celecoxib, an selective COX-2 inhibitor, and NSAID can also reduce the occurrence of digestive system cancers, such as inhibiting the proliferation of gastric, esophageal, and colorectal cancer cells[47,48]. It is estimated that long-term use of NSAIDs can reduce the incidence of colon cancer by 40%-50% [49]. However, studies have shown that the use of NSAIDs is not an effective chemoprophylaxis for all cancer patients, as aspirin has no effect on the incidence of colorectal adenoma or cancer in patients with Lynch syndrome[50]. Therefore, the combined use of COX-2 inhibitors and the development of new inhibitors have gradually emerged and have better antitumor activity. More detailed results are shown in Table 2. Moreover, clinical use of COX-2 inhibitors to prevent or treat gastric cancer may be limited because of potential side effects, especially in the cardiovascular system, such as elevated blood pressure and myocardial infarction[51,52]. Recently, a systematic review of 329 studies suggested that in addition to COX-2-selective inhibitors, NSAIDs also increase the risk of cardiovascular morbidity[53]. These side effects and low

treatment efficacy hinder the application of NSAIDs and COX-2 selective inhibitors as chemopreventive drugs to prevent cancer. At the same time, a study indicated that the combined regulation of the inflammatory microenvironment by inhibiting the COX-2/PGE2 and TLR/MyD88 pathways may be an effective strategy to prevent or treat the development and malignant progression of gastrointestinal cancer, especially those with p53 gain-of-function mutations[54]. Therefore, targeting the COX-2/PGE2 pathway combined with TLR/MyD88 signal pathway may inhibit the inflammatory microenvironment and the stemness of gastric tumor cells, which may be an effective strategy for the prevention and treatment of gastric cancer and needs further clinical evaluation[26]. In addition, as the information of genetic susceptibility and *COX-2* SNPs may have the potential to establish risk stratified markers in the general population, it is helpful for early screening and treatment of precancerous lesions in high-risk population groups to reduce the incidence of gastric cancer and avoid unnecessary treatment.

CONCLUSION

It has been established that the expression of COX-2 in gastric cancer cells is induced by various pathways including *H. pylori* infection and COX-2 overexpression results in the generation of an inflammatory microenvironment to promote the occurrence of gastric carcinomas. The polymorphisms including rs689466G>A, rs20417G>C, rs3218625G>A, rs5275T>C, and rs689470T>C in *COX-2* confer individuals a higher susceptibility to gastric cancer. NSAIDs can reduce the risk of digestive system malignant tumors. In addition, the combined regulation of the COX-2/PGE2 and TLR/MyD88 signaling pathways may be an effective strategy to prevent or treat the occurrence and development of gastrointestinal tumors. However, these treatments may increase the incidence of cardiovascular diseases. The above results encourage further functional research to find more accurate individualized prevention strategies and better therapies for gastric cancer.

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ORIGINAL ARTICLE

Basic Study

Y-box binding protein 1 augments sorafenib resistance *via* the PI3K/Akt signaling pathway in hepatocellular carcinoma

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Author contributions: Liu T, Xie XL and Jiang HQ conceived, designed the study; Liu T, Xie XL, Zhou X, Chen SX and Wang YJ performed most experiments, analyzed the data, wrote the manuscript and edited the paper; Xie XL and Jiang HQ helped to supervised the study; Shi LP, Chen SJ, Wang YJ, Wang SL, Zhang JN, Dou SY, Cui RL and Jiang XY helped to perform the experiments and analyzed the data; Xie XL and Jiang HQ helped to edited the paper.

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Abstract

BACKGROUND

Sorafenib is the first-line treatment for patients with advanced hepatocellular carcinoma (HCC). Y-box binding protein 1 (YB-1) is closely correlated with tumors and drug resistance. However, the relationship between YB-1 and sorafenib resistance and the underlying mechanism in HCC remain unknown.

AIM

To explore the role and related mechanisms of YB-1 in mediating sorafenib resistance in HCC.

METHODS

The protein expression levels of YB-1 were assessed in human HCC tissues and adjacent nontumor tissues. Next, we constructed YB-1 overexpression and knockdown hepatocarcinoma cell lines with lentiviruses and stimulated these cell lines with different concentrations of sorafenib. Then, we detected the proliferation and apoptosis in these cells by terminal deoxynucleotidyl transferase

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dUTP nick end labeling, flow cytometry and Western blotting assays. We also constructed a xenograft tumor model to explore the effect of YB-1 on the efficacy of sorafenib *in vivo*. Moreover, we studied and verified the specific molecular mechanism of YB-1 mediating sorafenib resistance in hepatoma cells by digital gene expression sequencing (DGE-seq).

RESULTS

YB-1 protein levels were found to be higher in HCC tissues than in corresponding nontumor tissues. YB-1 suppressed the effect of sorafenib on cell proliferation and apoptosis. Consistently, the efficacy of sorafenib *in vivo* was enhanced after YB-1 was knocked down. Furthermore, KEGG pathway enrichment analysis of DGE-seq demonstrated that the phosphoinositide-3-kinase (PI3K)/protein kinase B (Akt) signaling pathway was essential for the sorafenib resistance induced by YB-1. Subsequently, YB-1 interacted with two key proteins of the PI3K/Akt signaling pathway (Akt1 and PIK3R1) as shown by searching the BioGRID and HitPredict websites. Finally, YB-1 suppressed the inactivation of the PI3K/Akt signaling pathway induced by sorafenib, and the blockade of the PI3K/Akt signaling pathway by LY294002 mitigated YB-1-induced sorafenib resistance.

CONCLUSION

Overall, we concluded that YB-1 augments sorafenib resistance through the PI3K/Akt signaling pathway in HCC and suggest that YB-1 is a key drug resistance-related gene, which is of great significance for the application of sorafenib in advanced-stage HCC.

Key Words: Y-box binding protein 1; Hepatocellular carcinoma; Sorafenib; Drug resistance; Phosphoinositide-3-kinase/protein kinase B

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Core Tip: Y-box binding protein 1 (YB-1) was significantly increased in hepatocellular carcinoma (HCC), and it could increase the IC_{50} values of sorafenib in HCC cell lines. Meanwhile, YB-1 suppressed apoptosis and cell proliferation inhibition induced by sorafenib. Furthermore, we screened the phosphoinositide-3-kinase (PI3K)/protein kinase B (Akt) signaling pathway to explore the molecular mechanism of sorafenib resistance by the KEGG pathway enrichment analysis of the digital gene expression profiling-seq. And the blockade of PI3K/Akt signaling pathway by LY294002 mitigated YB-1-induced sorafenib resistance. Given that sorafenib is the first-line treatment for patients with advanced HCC, we proposed that the down-regulation of YB-1 is of great significance for the application of sorafenib in advanced HCC.

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INTRODUCTION

Approximately 90% of liver cancer cases are hepatocellular carcinoma (HCC)[1,2]. Approximately 420,000 people die of HCC every year in China, a region with a high HCC incidence[3,4]. Although studies have tried to clarify the pathogenesis of HCC, our understanding of HCC remains limited[5,6], and treatments including resection, ablation or transplantation are only feasible for patients diagnosed early[2,7]. For those with advanced HCC, the best treatment options are still lacking. The Food and Drug Administration (FDA) has approved sorafenib, which is a multitarget tyrosine kinase inhibitor, as a first-line drug for patients with advanced HCC and provides circumscribed survival benefits[8-10]. Additionally, because HCC is prone to relapse and

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metastasis, the prognosis of this refractory disease remains frustrating[11]. Therefore, further deepening our understanding of the molecular mechanisms of HCC and developing new intervention strategies are critical.

Y-box binding protein 1 (YB-1) is a multifunctional protein of the Y-box binding protein family that is distributed in both the cytoplasm and nucleus[12]. Current research on the function of YB-1 mainly involves the following three aspects: (1) YB-1 promotes the proliferation of hepatoma cells *via* the Wnt (wingless/integrated)/β-catenin signaling pathway[13] and promotes human breast epithelial tumor cell proliferation by activating the endothelial growth factor receptor (EGFR)/protein kinase B (Akt) signaling pathway[14]; (2) YB-1 effectively inhibits the gene expression of fatty acid synthase (Fas), thereby preventing the occurrence of apoptosis[15], and binds to the key apoptotic factor p53 to form protein complexes, which also exert anti-apoptotic effects[16,17]; (3) YB-1 activates the expression of multidrug resistance (MDR) genes in multiple cancers, such as kidney cancer, breast cancer and colorectal cancer[18]. Additionally, the most recent research indicates that YB-1 augments gefitinib resistance by activating the Akt signaling pathway and epithelial-mesenchymal transition (EMT) by targeting major vault proteins in lung adenocarcinoma cells[19]. Furthermore, YB-1 is significantly increased in pancreatic cancer, breast cancer, colorectal cancer, melanoma and sarcoma[13,14,20]. Chao *et al*[13] proved that the YB-1 expression level is significantly elevated in HCC and closely related to drug resistance. However, the specific underlying molecular mechanism remains uncertain. Therefore, our study intends to elucidate the relationship between YB-1 and sorafenib resistance in HCC and further explore the specific molecular mechanism underlying sorafenib resistance.

MATERIALS AND METHODS

Patients and samples

HCC tissues and paired paracancerous tissues (normal) were surgically resected from 6 patients with HCC and were obtained from 09/2020 to 11/2020 at our hospital. Patients with HCC diagnosed according to clinical and histological diagnostic criteria for HCC in China[21] were included in this study, and patients with tumor recurrence or other organ tumors were excluded. The diagnosis of HCC and histological evaluation were performed by an experienced pathologist. After excision, the tissues were fixed with 4% paraformaldehyde (Shijiazhuang Huawo Kerui Biological Technology Co., Ltd., Hebei Province, China) or immediately placed at -80 °C. All the patients signed informed consent forms, and the Ethics Committee of the Second Hospital of Hebei Medical University approved our study (approval letter No. 2020-P025).

Cell culture

The human normal hepatic cell line (LO2) and human hepatocarcinoma cell lines (HepG2, Huh7, Bel7402, SMMC7721) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, United States). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, United States) supplemented with 10% fetal bovine serum (FBS, HyClone, South Logan, UT, United States), 100 μg/mL of streptomycin and 100 U/mL of penicillin (Gibco BRL). Overexpression was accomplished by transfection with lentivirus-YB-1 (Lv-YB-1) for 24 h (Shanghai GenePharma Co., Ltd., Shanghai, China, Supplementary Table S1). Sequences of the YB-1 siRNA are displayed in Supplementary Table S2. Lentiviral vectors harboring YB-1-specific shRNA (Lv-shYB-1) were used (Shanghai GenePharma Co., Ltd, Supplementary Table S1), and cells were transfected for 24 h. Hepatoma cells were then incubated with 2.5 or 5 μmol/L sorafenib (MedChemExpress, Monmouth Junction, NJ, United States) for 24-48 h. LY294002 [a broad-spectrum inhibitor of phosphoinositide-3-kinase (PI3K), 20 μmol/L, 24 h] (MedChemExpress) or vehicle was also used to incubate YB-1-overexpressing cells with sorafenib.

Cell viability assay

Cell Counting Kit-8 (CCK-8) assays (Shanghai Share-bio Biotechnology Co., Shanghai, China) were used to examine the half-inhibitory concentration (IC_{50}). Briefly, the cells were seeded into 96-well plates at a density of 5×10^3 cells/well and incubated with different concentrations of sorafenib. Next, CCK-8 (10 μL/well) was added (serum-free medium *vs* CCK-8 = 10:1) to a volume of 110 μL and incubated for 2 h. The optical density (OD) value was measured using a microplate reader (BioTek, Winooski, VT,

United States) at 450 nm. For the cell cycle distribution analysis, the cells were cultured in six-well plates and collected when the cells were transfected and incubated for 24 h. Hereafter, we washed the cells with precooled phosphate-buffered saline (PBS; Solarbio Science and Technology Co., Ltd., Beijing, China), fixed the cells in 70% ethanol (Tianjin YongDa Chemical Reagent Co., Ltd., Tianjin, China), and subjected them to staining with PI/RNase Staining Solution (Beyotime Biotechnology Co., Shanghai, China) at 37 °C in the dark for 30 min. A FACSVerse flow cytometer (BD Biosciences, San Jose, CA, United States) was used to analyze the samples, and ModFit 5.0 software (BD Biosciences) was used to process the data.

Apoptosis assays

Following treatment, we collected and washed the cells with precooled PBS and utilized the Annexin V Apoptosis Detection Kit (Invitrogen, Carlsbad, CA, United States) to detect apoptotic cells. Briefly, the cells were incubated with staining solution containing 2.5 μL of Annexin V-APC, 5 μL of propidium iodide (PI) buffer and 200 μL of binding buffer for 20 min in the dark at room temperature. A FACSVerse flow cytometer was applied to analyze the samples. Additionally, a one-step terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis detection kit (Beyotime Biotechnology Co.) was employed to examine apoptosis according to the manufacturer's instructions. TUNEL-positive cells were recorded as the measurement of cell apoptosis. A laser scanning confocal microscope (Olympus, Tokyo, Japan) was used to acquire representative images.

Animal experiments

BALB/c nude mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Approximately 2 × 10^6 Huh7 cells transfected with Lv-shNC or Lv-shYB-1 were subcutaneously injected into male BALB/c nude mice aged 4 wk. We measured the tumors every 2 d, and (length × width^2)/2 was used to calculate the tumor volume. Next, we randomly divided the mice into designated groups (n = 6) when the average volume of the tumors reached approximately 100 mm^3. The nude mice in the Lv-shNC + sorafenib and Lv-shYB-1 + sorafenib groups were treated with sorafenib by gavage at a dosage of 30 mg/kg/d for 14 d. We sacrificed the nude mice when the treatment was completed, and the tissues were fixed with 4% paraformaldehyde or immediately placed at -80 °C. The animal protocol was designed to minimize pain and discomfort to animals. The nude mice were acclimatized to laboratory conditions for 1 wk prior our experiment. Intragastric gavage administration was conducted by using straight gavage needles (22 gauge). All the animals in our study received humane care, and the ethics committee of the Second Hospital of Hebei Medical University approved our study (approval letter No. 2020-AE002).

Histological and immunohistochemical analyses

We fixed human liver tissues and mouse xenograft tumors with 4% paraformaldehyde, embedded tissues in paraffin (Shanghai YiYang Instrument Co., Ltd., Shanghai, China), and sectioned them. The sections were then subjected to hematoxylin and eosin (HE, Beijing Legian Biotechnology Co., Ltd., Beijing, China) and immunohistochemical (IHC) staining using standard protocols as previously described[22]. YB-1 (diluted 1:200; catalog number: CY5462; Shanghai Abways Biotechnology Co., Ltd., Shanghai, China) and proliferating cell nuclear antigen (PCNA; diluted 1:200; catalog number: AB0051; Shanghai Abways Biotechnology Co., Ltd.) antibodies were used in our study, and IHC images were obtained using an upright microscope (Leica, Wetzlar, Germany). We utilized ImageJ software (National Institutes of Health, Bethesda, MD, United States) to morphometrically evaluate the percentage of positive areas on the micrographs.

Western blot analysis

Cells or tissues were lysed using radioimmunoprecipitation assay buffer [50 mmol/L Tris, 1 mmol/L ethylenediaminetetraacetic acid, 150 mmol/L NaCl, 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% sodium deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride] (Beyotime Biotechnology Co.) for total protein extraction after washing in PBS. Next, we quantified the protein concentrations by Coomassie blue staining (Solarbio Science and Technology Co., Ltd.) and denatured in SDS loading buffer (Beyotime Biotechnology Co.) by boiling for 10 min. The proteins from each sample were subjected to SDS-polyacrylamide gel electrophoresis (Shanghai Epizyme Biotechnology Co., Ltd., Shanghai, China) and transferred onto polyvinylidene difluoride membranes (Merck Millipore, Billerica, MA, United States). Next, 5%

milk (Solarbio Science and Technology Co., Ltd.) was used to block the membranes for 1 h, followed by incubation with the primary antibodies overnight at 4 °C. The next day, the membranes were incubated with fluorescence-conjugated secondary antibodies (LI-COR Biosciences, Lincoln, NE, United States) for 1 h at room temperature. We applied an Odyssey infrared imaging system (LI-COR Bioscience) to visualize the bands and quantified them using ImageJ software. GAPDH, PI3K or Akt was applied to normalize the desired bands, and the results were shown as relative density ratios. The primary antibodies used included YB-1 (diluted 1:500), p-PI3K (diluted 1:100; catalog number: AF3242; RRID: AB_2834668; Affinity Biosciences Ltd., Jiangsu, China), PI3K (diluted 1:100; catalog number: AF6242; RRID: AB_2835106; Affinity Biosciences Ltd.), GAPDH (diluted 1:1000; catalog number: AB0037; Shanghai Abways Biotechnology Co., Ltd.), p-Akt (diluted 1:100; catalog number: CY6569; Shanghai Abways Biotechnology Co., Ltd.), Akt (diluted 1:500; catalog number: CY5561; Shanghai Abways Biotechnology Co., Ltd.), PCNA (diluted 1:1000), and PARP (diluted 1:1000; catalog number: #9532; RRID: AB_659884; Cell Signaling Technology, Danvers, MA, United States).

Digital gene expression profiling

Two samples of HepG2 cell lines (Lv-shNC + sorafenib and Lv-shYB-1 + sorafenib) with stable YB-1 knockdown and sorafenib treatment were used, and each sample was repeated 3 times. Next, we submitted the samples to Beijing Novogene Technology Co., Ltd. (Beijing, China) for digital gene expression profiling (DGE) sequencing analysis to screen out DEGs. The analysis process mainly included data quality control, reference genome comparison, quantitative analysis, significance analysis of differences and functional enrichment analysis. The DESeq2 R package (1.16.1) was employed to perform the differential expression analysis between the groups (three biological replicates per group). The Benjamini and Hochberg approach to control the false discovery rate was adopted to adjust the resulting *P*-values (padj). A padj less than 0.05 and an absolute value of the log2 (fold change) more than 0 were utilized as the thresholds to recognize the differentially expressed genes[23,24].

Statistical analysis

All the experiments were repeated three times. Normally distributed data were expressed as mean ± SD, and nonnormally distributed data were expressed as medians with interquartile range. The difference between two groups was analyzed by Student's *t*-test. For differences among multiple groups, one-way ANOVA was performed. SPSS software version 19.0 (IBM, Armonk, NY, United States) was used to perform all statistical analyses. *P* values less than 0.05 were deemed statistically significant.

RESULTS

YB-1 is upregulated in HCC samples and cell lines

To determine the role of YB-1 in HCC, the expression of YB-1 in 6 paired HCC tissues and paracarcinoma tissues was detected by Western blot analysis. The YB-1 protein levels in HCC tissues were significantly elevated in all 6 patients (Figure 1A). To further verify the dysregulation of YB-1, we also performed IHC to detect YB-1 expression in the 6 paraffin-embedded HCC tissues and their paired adjacent liver tissues. The protein levels of YB-1 were also higher in HCC tissues than in nontumor tissues, and YB-1 was mainly located in the cytoplasm (Figure 1B, C). The expression of YB-1 was further detected in several hepatocarcinoma cell lines. Compared with the protein levels of YB-1 in LO2 cells, those in HepG2 and Huh7 cells were dramatically increased but slightly elevated in Bel7402 and SMMC7721 cells ($P < 0.05$) (Figure 1D). Taken together, YB-1 is elevated in HCC tissues and cell lines, implying that it might play a role in tumor progression.

YB-1 induces sorafenib resistance in hepatoma cells

Recent studies have shown that YB-1 is related to anticancer drug resistance in multiple tumor types. The IC_{50} of sorafenib in HCC cell lines was tested, and the IC_{50} values of Bel7402, SMMC7721, HepG2 and Huh7 cells were 4.47 μmol/L, 3.98 μmol/L, 5.01 μmol/L, and 2.51 μmol/L, respectively (Supplementary Figure S1A). We also found that YB-1 protein levels were upregulated in Bel7402, SMMC7721, HepG2 and Huh7 cells 24 h after sorafenib treatment (Supplementary Figure S1B). The transfection

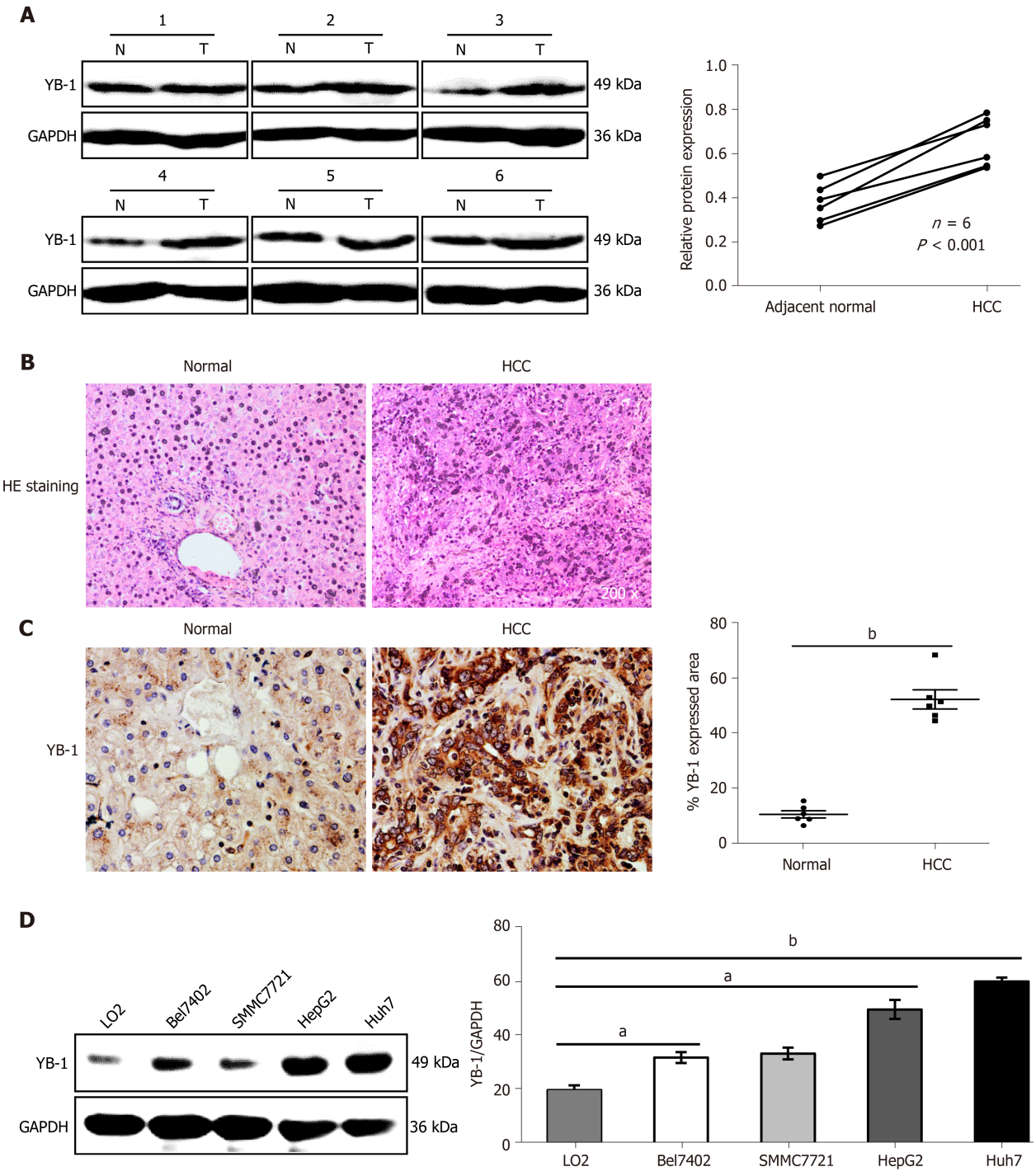


Figure 1 Increased expression of Y-box binding protein 1 in the liver tissues of hepatocellular carcinoma patients and cell lines. A: The protein expression levels of Y-box binding protein 1 (YB-1) in 6 pairs of hepatocellular carcinoma (HCC) liver tissues and their corresponding nontumor tissues were detected by Western blot analysis. Paraffin-embedded sections of liver tissues were stained with hematoxylin-eosin (HE) (B), and immunohistochemistry staining of YB-1 (C) was performed in the 2 groups; D: Western blot analysis showed YB-1 protein expression in one normal (N) liver cell line and four HCC cell lines. YB-1 protein levels (normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) were measured by scanning densitometry. $^{a}P < 0.05$, $^{b}P < 0.001$ *vs* the indicated groups. T: Tumor.

efficiency of the YB-1 siRNA and lentivirus is displayed in Supplementary Figure S2. We further tested the IC_{50} values of sorafenib in HepG2 and Huh7 cells with overexpressed or knocked down YB-1. The IC_{50} values were increased after YB-1 overexpression, whereas they were lower in the two cell lines with Lv-shYB-1 (Figure 2A).

Hereafter, we elucidated the effects of YB-1 and sorafenib on cell proliferation and apoptosis in HepG2 and Huh7 cells. Overexpressing YB-1 decreased the number of G1-phase cells and increased the number of S-phase cells, and sorafenib restrained the increase in G1-phase cells and reduced the number of S-phase cells (Figure 2B, C). Simultaneously, YB-1 silencing resulted in increased G1-phase and reduced S-phase

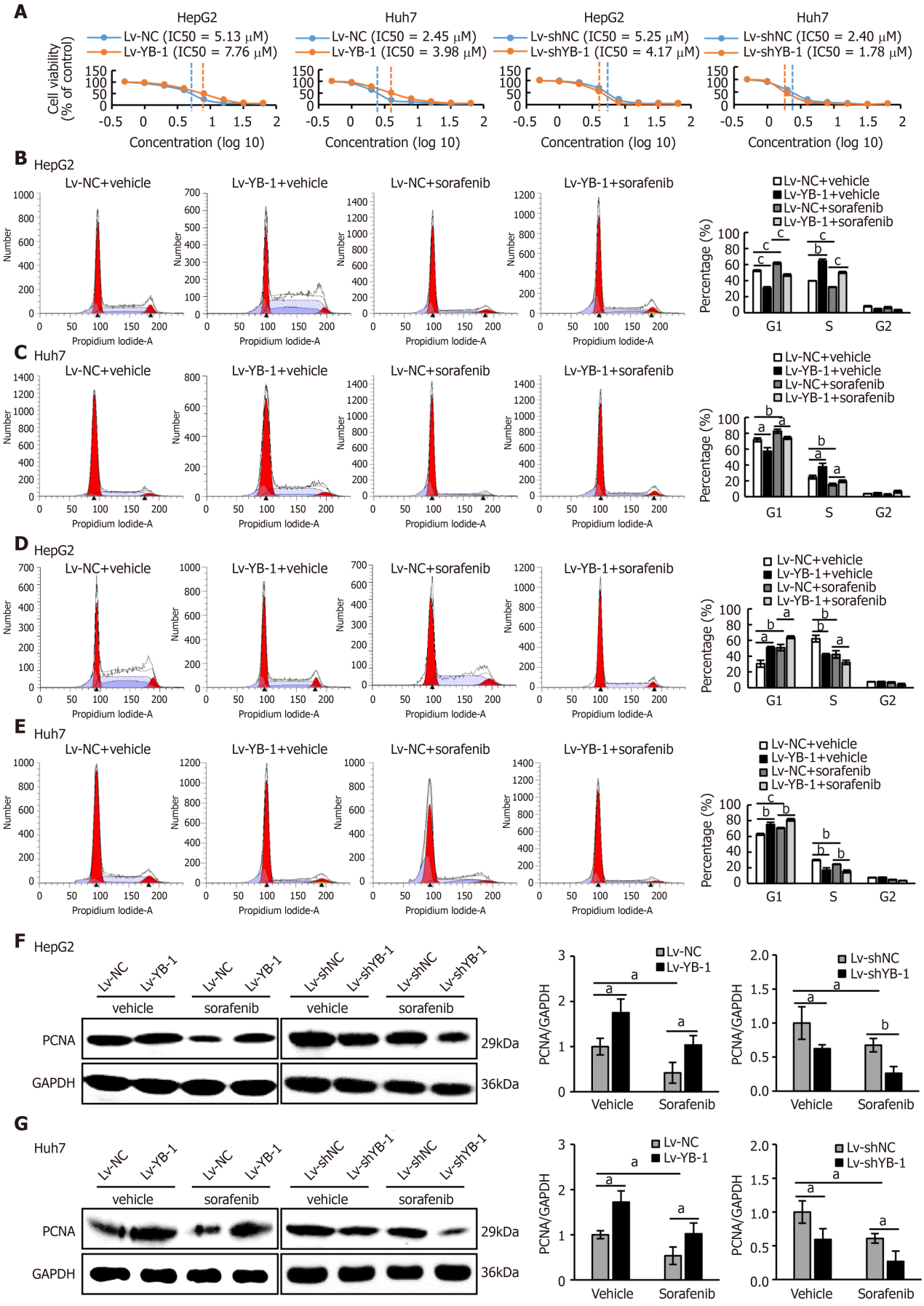


Figure 2 Y-box binding protein 1 offsets the effect of sorafenib on proliferation in hepatoma cell lines. A: The IC_{50} values of sorafenib in HepG2 and Huh7 cells infected with lentivirus encoding the Y-box binding protein 1 (lv-YB-1) or lentivirus encoding short hairpin RNA targeting YB-1 (Lv-shYB-1) were

detected by the Cell Counting Kit-8 (CCK-8) assay; B-E: Flow cytometry was conducted to perform cell cycle distribution analysis of the cells infected with the empty lentivirus vector as a negative control (Lv-NC) ± sorafenib *vs* Lv-YB-1 ± sorafenib groups (B, C) and lentivirus containing non-specific short hairpin RNA as a negative control (Lv-shNC) ± sorafenib *vs* Lv-shYB-1 ± sorafenib groups (D, E); F, G: Western blot analysis showed proliferating cell nuclear antigen (PCNA) protein expression in the Lv-NC ± sorafenib *vs* Lv-YB-1 ± sorafenib groups and Lv-shNC ± sorafenib *vs* Lv-shYB-1 ± sorafenib groups of HepG2 (F) and Huh7 (G) cells. PCNA protein levels (normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) were measured by scanning densitometry. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* the indicated groups.

cells and enhanced the effect of sorafenib on G1-phase and S-phase cells (Figure 2D, E). Additionally, we examined the levels of PCNA and found that it was increased in cells with YB-1 overexpression that the YB-1 overexpression offset PCNA reduction caused by sorafenib and that the knockdown of YB-1 had opposite effects in HepG2 (Figure 2F) and Huh7 cells (Figure 2G). These results indicated that YB-1 played a very important role in the G1/S transition in sorafenib-resistant HCC cell lines. Next, apoptosis was analyzed by TUNEL and flow cytometric analysis in HepG2 and Huh7 cells. YB-1 overexpression decreased cell apoptosis and reduced apoptosis induced by sorafenib (Figure 3A-D), while YB-1 knockdown strengthened the effect of sorafenib in HepG2 and Huh7 cells (Figure 4A-D). Furthermore, the protein levels of cleaved PARP decreased after YB-1 overexpression, which reduced the sorafenib-induced protein expression of activated PARP (Figure 3E, F). However, YB-1 knockdown played an opposite role in HepG2 and Huh7 cells (Figure 4E, F). Overall, these data proved that YB-1 mediated sorafenib resistance in liver cancer cell lines.

YB-1 knockdown ameliorates the efficiency of sorafenib in vivo

Xenograft tumor models were constructed using Huh7 cells with stable YB-1 knockdown. Tumor growth was suppressed both by YB-1 knockdown and sorafenib treatment. Nevertheless, the most obvious suppression was achieved by the amalgamation of YB-1 knockdown and sorafenib therapy (Figure 5A, B and Supplementary Figure S3). Additionally, YB-1 knockdown notably promoted the efficiency of sorafenib by examining tumor weights (Figure 5C). IHC staining also showed that the protein expression levels of PCNA in cells of the Lv-shYB-1 + sorafenib group were the lowest compared with those in cells with YB-1 knockdown or sorafenib treatment alone (Figure 5D). Furthermore, we extracted protein from xenograft tumors and found that the p-Akt and p-PI3K protein levels were downregulated after YB-1 knockdown or sorafenib treatment, and the most dramatic reduction occurred in the Lv-shYB-1 + sorafenib group (Figure 5E and F). Together, these results showed that YB-1 knockdown mitigated the resistance to sorafenib and reinforced the antitumor efficacy.

Identification of differentially expressed genes in the DGE-seq results of HepG2 cells

In total, 6254 significant differentially expressed genes were detected between the Lv-shYB-1 + sorafenib and Lv-shNC + sorafenib groups, among which 3317 were downregulated and 2937 were upregulated. The differentially expressed genes in the Lv-shYB1 + sorafenib and Lv-shNC + sorafenib groups were merged as the differential gene set. Mainstream hierarchical clustering was used to cluster the FPKM values (expected number of fragments per kilobase of transcript sequence per million base pairs sequenced) of the differential gene set to gather genes with similar expression patterns. The hierarchical clustering heatmap (Figure 6A) showed that the expression patterns of genes in the Lv-shYB1 + sorafenib group were distinguishable from those of the Lv-shNC + sorafenib group.

GO and KEGG pathway analyses

We identified the functional categories and signaling pathways using GO and KEGG pathway analyses[25]. The differentially expressed genes were categorized into three major GO categories: biological process (BP), cellular component (CC) and molecular function (MF). The top 30 GO categories are shown in Figure 6B. The differentially expressed genes were mapped to the reference canonical pathways using KEGG. The top 20 KEGG pathways were demonstrated in Figure 6C. Additionally, we searched the BioGRID (https://thebiogrid.org/) and HitPredict (www.hitpredict.org/) websites to explore the potential YB-1-interacting proteins. In the top 20 KEGG pathways, we found two key proteins that interact with YB-1 in the PI3K/Akt signaling pathway: (1) Akt1: activated Akt binds to the YB-1 cold shock domain at Ser102[26,27]; and (2) phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1): PI3K is a critical regulator in

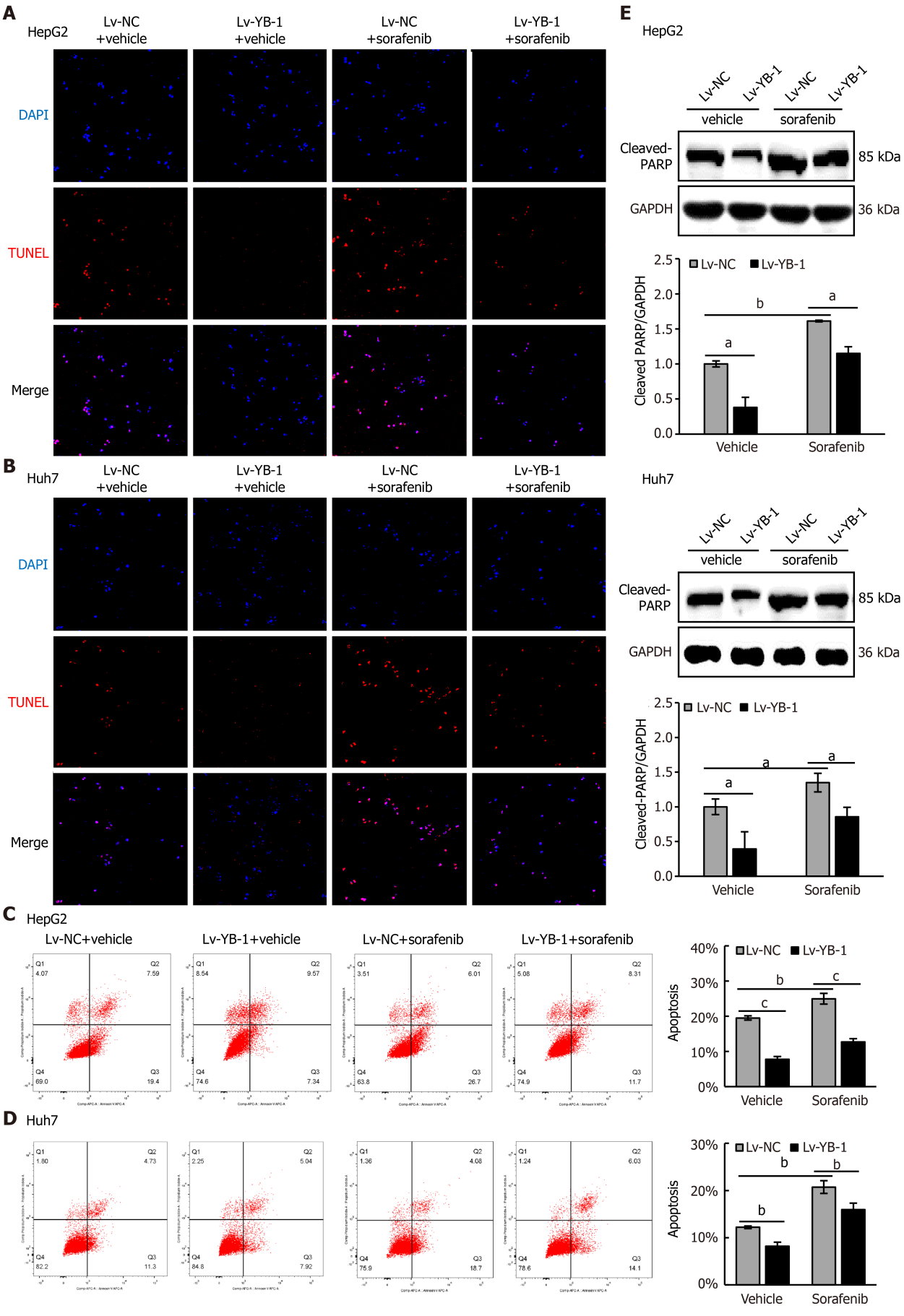


Figure 3 Y-box binding protein 1 suppresses apoptosis induced by sorafenib treatment in hepatocellular carcinoma cell lines. A-D:

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and flow cytometry were employed to analyze apoptosis in the cells infected with empty lentivirus vector as a negative control (Lv-NC) ± sorafenib *vs* lentivirus with Y-box binding protein 1 (Lv-YB-1) ± sorafenib groups; E, F: Western blot analysis showed cleaved poly ADP-ribose polymerase (PARP) protein expression in the Lv-NC ± sorafenib/Lv-YB-1 ± sorafenib groups. Cleaved PARP protein levels (normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) were measured by scanning densitometry. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* the indicated groups. DAPI: 4'6'-diamidino-2-phenylindole dihydrochloride.

the PI3K/Akt pathway, and YB-1 is one of the 49 cross-species PI3K binding proteins from Drosophila-human reciprocal blasts[28]. Pathway enrichment analysis indicated that 133 differentially expressed genes were annotated to the PI3K/Akt pathway. Additionally, our results suggest that the mRNA expression of Akt1 and Akt2 was significantly changed after YB-1 knockdown (Figure 6D). Furthermore, the Akt signaling pathway correlates with YB-1[29], sorafenib treatment[30] and HCC[31]. Therefore, we planned to further investigate the potential relationship of YB-1 and the PI3K/Akt pathway in HCC cell lines treated with sorafenib.

YB-1 promotes sorafenib resistance via the PI3K/Akt signaling pathway

Western blot analysis was consequently applied to detect the expression levels of key proteins in the PI3K/Akt signaling pathway in HepG2 and Huh7 cells. The expression levels of p-Akt and p-PI3K were increased in YB-1-overexpressing cells, and sorafenib decreased the protein expression; however, the association of YB-1 overexpression and sorafenib treatment neutralized the expression of the two proteins (Figure 7A-D). Simultaneously, either single knockdown of YB-1 or treatment with sorafenib inhibited p-Akt and p-PI3K expression. Furthermore, the most significant suppression of protein expression was accomplished by the combination of YB-1 knockdown and sorafenib incubation (Figure 7A-D).

Next, we explored whether blockade of the PI3K/Akt pathway would avoid YB-1-induced sorafenib resistance in Lv-NC + sorafenib and Lv-YB-1 + sorafenib-treated HepG2 and Huh7 cells. The phosphorylation of PI3K and Akt was obviously attenuated after the suppression of PI3K/Akt signaling by LY294002 in Lv-NC and Lv-YB-1 cells incubated with sorafenib (Figure 7E-H). Moreover, LY294002 led to increased G1-phase and reduced S-phase cells, inhibited YB-1-induced decreased G1-phase and increased S-phase cells (Figure 7I and J), and decreased PCNA expression (Figure 7K-M). Simultaneously, the suppression of PI3K/Akt signaling by LY294002 increased apoptosis in Lv-NC and Lv-YB-1 cells with sorafenib incubation (Figure 8A-D) and elevated the protein levels of cleaved PARP (Figure 8E and F). Taken together, YB-1 induced sorafenib resistance by activating the PI3K/Akt signaling pathway.

DISCUSSION

Researchers have mainly focused on the relationship between YB-1 and tumor proliferation, apoptosis, invasion, metastasis and other biological behaviors[32-36]. To date, few studies have investigated the correlation between YB-1 and drug resistance. In addition to YB-1 activating MDR in various tumors[18], YB-1 also induces the development of chemotherapy resistance by regulating the expression of P-glycoprotein and focal adhesion kinase and the characteristics of stem cells in various tumors[37-39]. Additionally, indirubin 3'-oxime inhibits the nuclear translocation of YB-1, thereby reducing MDR1 expression and exerting efficacy in treating liver cancer [40]. Sorafenib, a new type of multitarget antitumor drug, is a first-line systemic treatment option for patients with advanced HCC[41]. It has dual antitumor effects: (1) it blocks the signaling pathway mediated by RAF/MEK/extracellular regulated protein kinases (ERK) and directly inhibits tumor cell proliferation; (2) it inhibits vascular EGFR and platelet-derived growth factor receptor and then suppresses angiogenesis, indirectly inhibiting tumor cell growth[41]. Studies on the resistance mechanism of sorafenib have found that EMT, activation of the Akt signaling pathway, tumor stem cells, and abnormal regulation of proliferation and apoptosis induce sorafenib resistance[42].

The effect of sorafenib, which promotes cell apoptosis and inhibits cell proliferation, has been proven in previous studies[30,43,44]. A study on sorafenib resistance observed that β-catenin and its downstream target genes were elevated in hepatoma cells after incubation with sorafenib, and β-catenin overexpression inhibited apoptosis and promoted cell proliferation, while β-catenin knockdown enhanced the effect of sorafenib on apoptosis and proliferation. Additionally, the IC_{50} values of sorafenib

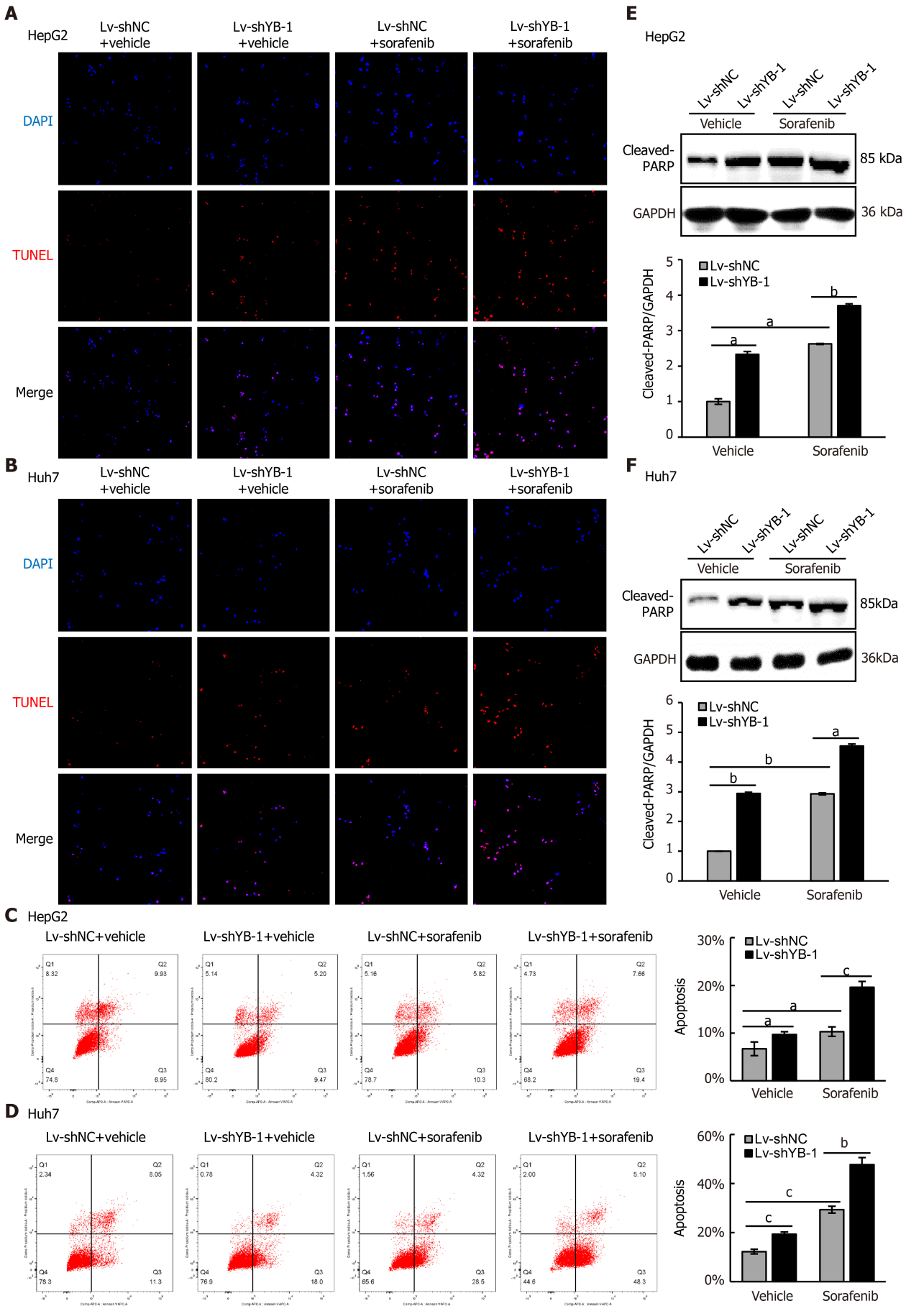


Figure 4 Y-box binding protein 1 suppresses apoptosis induced by sorafenib treatment in hepatocellular carcinoma cell lines. A-D:

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and flow cytometry were employed to analyze apoptosis in the cells infected with lentivirus encoding non-specific short hairpin RNA as a negative control (Lv-shNC) ± sorafenib *vs* lentivirus with short hairpin RNA targeting the Y box binding protein 1 (Lv-shYB-1) ± sorafenib groups; E, F: Western blot analysis showed cleaved poly ADP-ribose polymerase (PARP) protein expression in the Lv-shNC ± sorafenib/Lv-shYB-1±sorafenib groups. Cleaved PARP protein levels (normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) were measured by scanning densitometry. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* the indicated groups.

were heightened in liver cancer cells overexpressing β-catenin, confirming that β-catenin is closely related to sorafenib resistance. The relationship between YB-1 and sorafenib resistance in HCC was first clarified in our study. First, YB-1 in liver cancer tissues showed dramatically higher expression than that in the corresponding adjacent tissues. Next, we constructed lentiviruses that overexpress or knock down YB-1 expression in two human liver cancer cell lines (HepG2 and Huh7) and simultaneously stimulated cells with different concentrations of sorafenib; YB-1 significantly elevated the IC_{50} of HCC cells. We proved that the effect of sorafenib on apoptosis and proliferation was suppressed by YB-1 overexpression, while YB-1 knockdown had opposite effects. Uniformly, the association of YB-1 knockdown and sorafenib treatment had the most significant effect on the efficacy of sorafenib *in vivo*. These results indicate that YB-1 leads to sorafenib resistance in HCC[43,44].

Next, we conducted digital gene expression profile analysis in HepG2 cells (Lv-shNC + sorafenib, Lv-shYB-1 + sorafenib) with stable YB-1 knockdown and sorafenib treatment. Akt1 and Akt2 in the Lv-shYB-1 + sorafenib group were notably lower than those in the Lv-shNC + sorafenib group, and KEGG pathway enrichment analysis revealed a significant difference in the PI3K/Akt signaling pathway between the two groups. At the same time, we searched for proteins that interact with YB-1 using the BioGRID and HitPredict websites. Among proteins that interface with YB-1, we identified two proteins, Akt1[26,27] and PIK3R1[28], which are essential proteins of the PI3K/Akt signaling pathway.

The PI3K/Akt signaling pathway, a well-known survival mechanism of cancer, is tightly correlated with cancer occurrence and growth. Presently, studies on the PI3K/Akt signaling pathway has mostly focused on the proliferation, apoptosis, migration and invasion of tumor cells, such as gastric cancer[45], breast cancer[46], cervical squamous cell carcinoma[47] and osteosarcoma[48]. Additionally, YB-1 was reported to promote the tumorigenesis and progression of spinal chordoma by activating the EGFR/Akt signaling pathway[29]. At the same time, the PI3K/Akt pathway is also involved in chemotherapeutic drug resistance; for example, suppressing the expression of Akt sensitizes cells to sorafenib-induced apoptosis[49], and blocking the PI3K/Akt signaling pathway induces increased sensitivity of hepatocarcinoma cells to sorafenib[50-52]. Regarding acquired sorafenib resistance, the PI3K/Akt signaling pathway is considered a compensation mechanism, and multiple studies have revealed that the phosphorylation level of Akt in HCC cells with acquired sorafenib resistance is higher than that in its parental cells. Inhibition of Akt by GDC0068, MK-2206 or LY294002 reverses the occurrence of acquired sorafenib resistance[53-55]. Fortunately, we verified that YB-1 activates the PI3K/Akt signaling pathway and inhibits the inactivation of the PI3K/Akt signaling pathway induced by sorafenib treatment. Additionally, the blockade of PI3K/Akt signaling pathway by LY294002 inhibited YB-1-induced sorafenib resistance in HepG2 and Huh7 cells. Thus, YB-1 stimulates sorafenib resistance by targeting the PI3K/Akt signaling pathway in HCC.

CONCLUSION

In conclusion, the absence of YB-1 effectively suppresses the occurrence of sorafenib resistance and promotes the deactivation of the PI3K/Akt signaling pathway caused by sorafenib. Given that sorafenib is the first-line treatment for patients with advanced HCC, we propose that downregulation of YB-1 may be a novel potential approach to treat advanced HCC and improve the efficacy of sorafenib in treating advanced HCC.

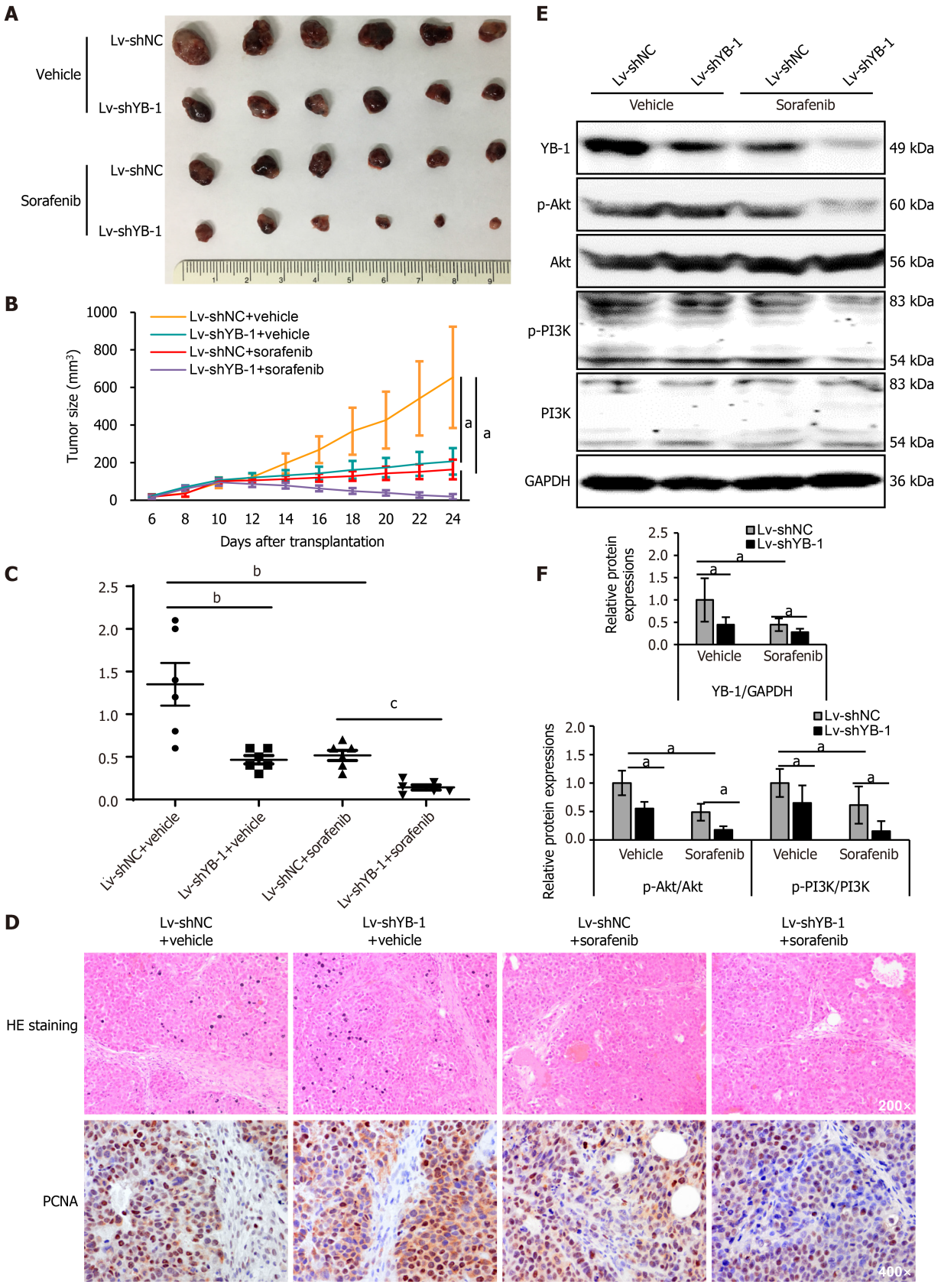


Figure 5 Sorafenib efficiency improves after Y-box binding protein 1 knockdown *in vivo*. A: The morphologies of collected tumors in subcutaneous Huh7 xenografts in nude mice; B: Tumor growth curves; C: Tumor weights were measured after collection; D: Hematoxylin-eosin (HE) and immunohistochemical staining for proliferating cell nuclear antigen; E, F: Western blot analysis showed Y-box binding protein 1 (YB-1), phosphorylated protein kinase B (p-Akt), protein kinase B (Akt), phosphorylated phosphoinositide-3-kinase (p-PI3K), and phosphoinositide-3-kinase (PI3K) expression in cells infected with lentivirus encoding non-specific short hairpin RNA as a negative control (Lv-shNC) ± sorafenib *vs* lentivirus containing short hairpin RNA targeting YB-1 (Lv-shYB-1) ± sorafenib groups. p-Akt (normalized to Akt) and p-PI3K (normalized to PI3K) protein levels were measured by scanning densitometry. Protein samples derived from the same experiment

and gels were processed in parallel. [a]*P* < 0.05, [b]*P* < 0.01, [c]*P* < 0.001 *vs* the indicated groups. PCNA: Proliferating cell nuclear antigen.

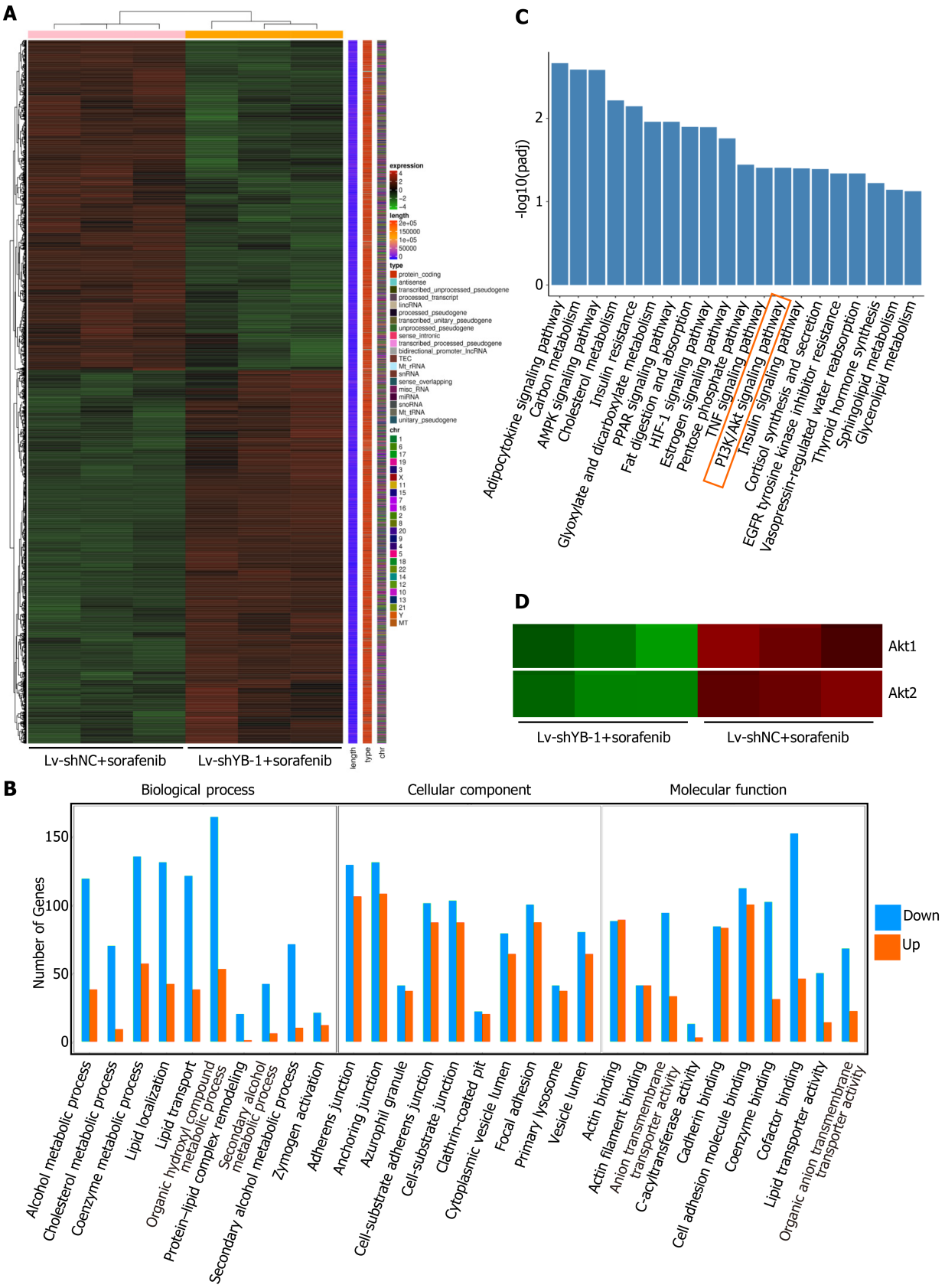
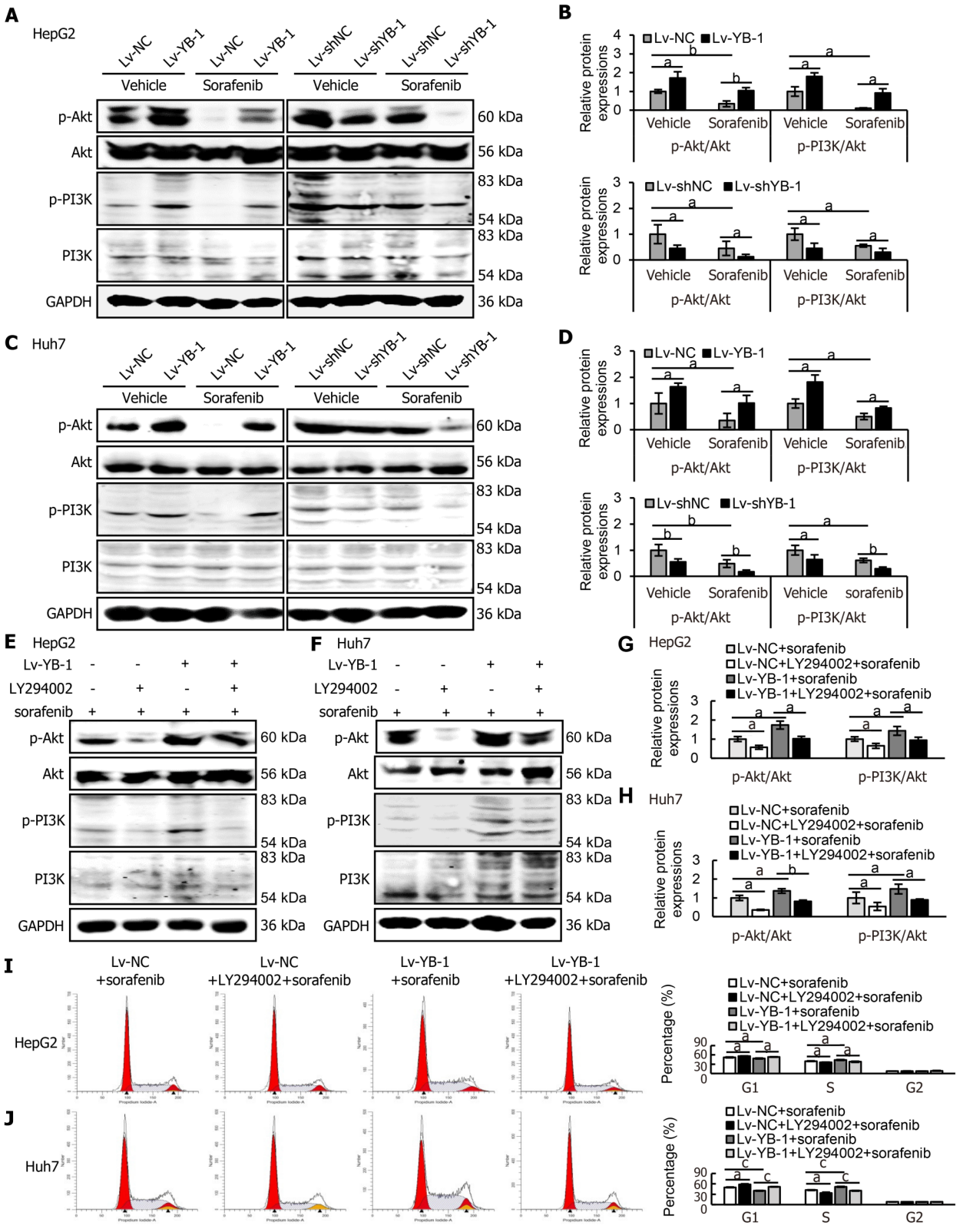


Figure 6 Digital gene expression profiling-seq analysis in HepG2 cells. A: Heat map of the results from the cluster analysis of the digital gene expression profiling (DGE)-seq data (cells infected with: lentivirus encoding non-specific short hairpin RNA as a negative control (Lv-shNC) + sorafenib *vs* lentivirus

encoding short hairpin RNA against Y box protein 1 (Lv-shYB-1) + sorafenib). Each column denotes the DEGs identified in our study. Each row indicates a sample. For each gene, red represents a high level of expression relative to the mean, while green expresses a low level. The scale bar is the number of standard deviations from the mean; B: Histogram of GO functional analysis for the DEGs obtained from DGE sequencing. GO terms with padj < 0.05 were thought to be notably enriched by DEGs. The y axis represents the number of genes in a GO classification category. Red represents increased expression in the Lv-shYB-1 + sorafenib group, whereas green indicates decreased expression in that group; C: Pathway enrichment analysis showed the top 20 signaling pathways in KEGG (padj < 0.05); D: mRNA expression levels of protein kinase B1 (Akt1) and protein kinase B2 (Akt2) from the DGE-seq data (Lv-shNC + sorafenib, Lv-shYB-1 + sorafenib).



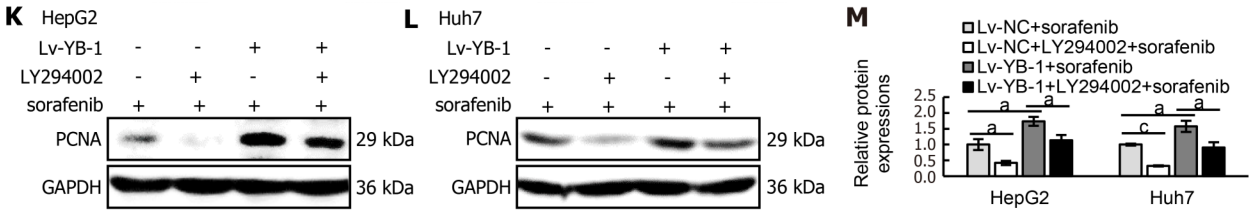
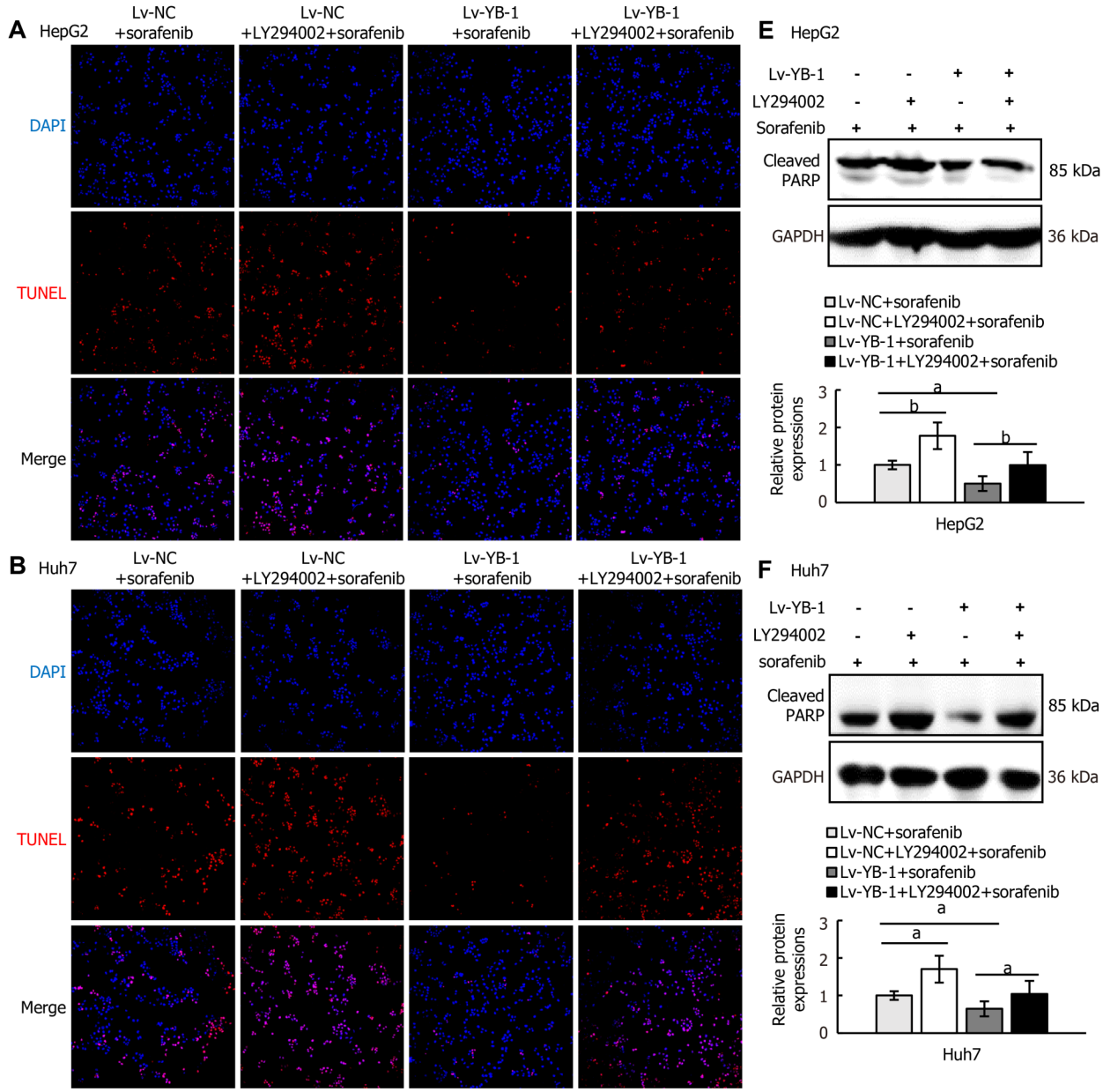


Figure 7 Y-box binding protein 1 suppresses the function of sorafenib in the PI3K/Akt signaling pathway, and blockade of the PI3K/Akt signaling pathway inhibits the promoting effect of YB-1 on proliferation. A-D: The protein expression levels of phosphorylated protein kinase B (p-Akt), protein kinase B (Akt), phosphoinositide-3-kinase (PI3K), and phosphorylated PI3K (p-PI3K) were detected by Western blot analysis in cells infected with lentivirus vector as a negative control (Lv-NC)±sorafenib *vs* cells infected with lentivirus encoding Y-box protein 1 (Lv-YB-1) ± sorafenib groups and lentivirus encoding non-specific short hairpin RNA as a negative control (Lv-shNC)±sorafenib *vs* cells infected with lentivirus encoding short hairpin RNA targeting Y-box binding protein 1 (Lv-shYB-1) ± sorafenib groups of HepG2 (A, B) and Huh7 (C, D) cells; E-H: Western blot analysis was also applied to evaluate the expression of p-Akt, Akt, p-PI3K and PI3K in the Lv-NC + sorafenib, Lv-NC + LY294002 + sorafenib, Lv-YB-1 + sorafenib, Lv-YB-1 + LY294002 + sorafenib groups of HepG2 (E, G) and Huh7 (F, H) cells. Protein samples derived from the same experiment and gels were processed in parallel; I, J: Flow cytometry was conducted to analyze the cell cycle distribution; K-M: Western blot analysis showed proliferating cell nuclear antigen (PCNA) protein expression, and its protein levels were measured by scanning densitometry. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* the indicated groups.



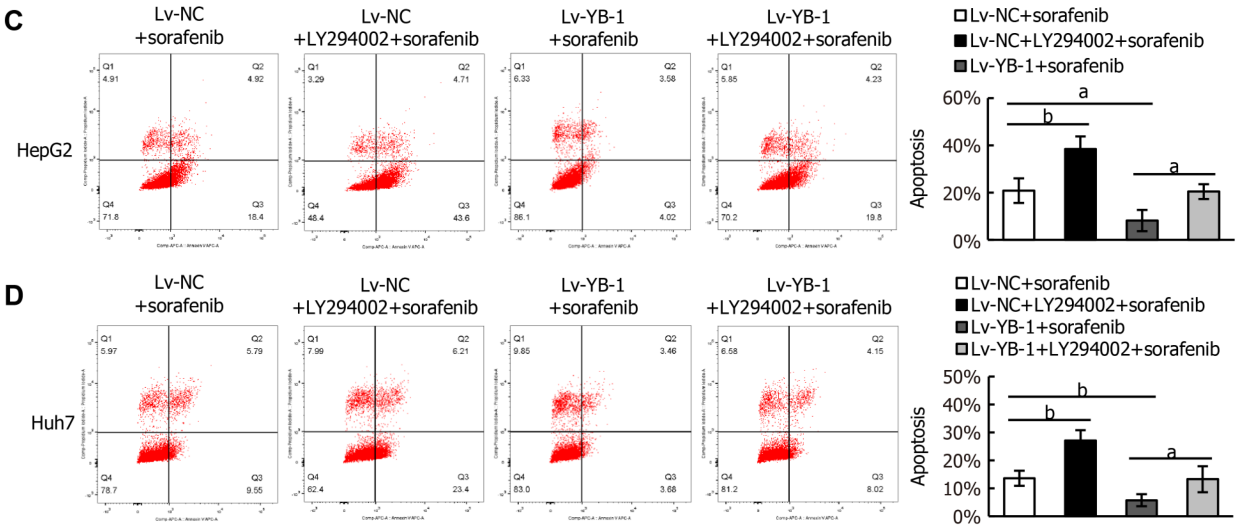


Figure 8 Blocking the PI3K/Akt signaling pathway suppresses the effect of Y-box binding protein 1 on apoptosis. A-D: Apoptosis analysis was conducted by terminal deoxynucleotidyl transferase dUTP nick end labeling staining (A, B) and flow cytometry with Annexin V-APC and PI staining (C, D); E, F: Western blot analysis showed cleaved poly ADP-ribose polymerase (PARP) protein expression. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* the indicated groups. Lv: Lentivirus; NC: Negative control; YB-1: Y-box binding protein 1; DAPI: 4'6'-diamidino-2-phenylindole dihydrochloride; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

ARTICLE HIGHLIGHTS

Research background

Y-box binding protein 1 (YB-1) promotes the proliferation of tumor cells, inhibits apoptosis, and activates the expression of multidrug resistance genes in breast cancer, kidney cancer and other cancers. It can also promote the resistance of gefitinib in lung adenocarcinoma cells. The specific molecular mechanism of YB-1 mediating drug resistance in hepatocellular carcinoma (HCC) is still unclear.

Research motivation

The study will provide a basis for the application of YB-1 in sorafenib resistance of HCC.

Research objectives

To detect the expression of YB-1 in HCC tissues and cells, and explore the role and the potential mechanism of YB-1 in sorafenib resistance of HCC.

Research methods

In this study, the authors examined the expression levels of YB-1 in cancer and corresponding paracancerous tissues of HCC patients, and clarified whether YB-1 was involved in sorafenib resistance in HCC by overexpressing and knocking down YB-1 in two kinds of hepatoma cell lines. Furthermore, a nude mouse liver cancer xenograft tumor model was established to verify the role of YB-1 in mediating sorafenib resistance in HCC *in vivo*. The signaling pathways involved in YB-1-mediated drug resistance in hepatocarcinoma cells were also screened and validated by digital expression profiling (DGE) technology.

Research results

YB-1 expression levels were significantly upregulated in both cancer tissues of HCC patients and human hepatoma cell lines. YB-1 could resist the effects of sorafenib on proliferation and apoptosis in hepatocarcinoma cells, which in turn mediated the resistance of HCC cells to sorafenib. YB-1 mediates sorafenib resistance in hepatocarcinoma cells through activating the phosphoinositide-3-kinase (PI3K)/protein kinase B (Akt) signaling pathway.

Research conclusions

Knockdown of YB-1 can effectively inhibit the occurrence of sorafenib resistance, and can also enhance the inactivation of sorafenib on the PI3K/Akt signaling pathway.

Therefore, YB-1 is a sorafenib resistance related gene.

Research perspectives

Downregulation of YB-1 may be a new potential method for the treatment of advanced HCC, and it is expected to improve the efficacy of sorafenib in the treatment of advanced HCC patients.

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ORIGINAL ARTICLE

Retrospective Study

Lens culinaris agglutinin-reactive fraction of alpha-fetoprotein improves diagnostic accuracy for hepatocellular carcinoma

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Institutional review board statement: This study protocol followed the ethical guidelines of the 1975 Declaration of Helsinki, and the institutional review board of Korea University Anam Medical Center.

Informed consent statement: The

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Abstract

BACKGROUND

Diagnostic accuracy of various tumor markers and their combinations for hepatocellular carcinoma (HCC) was not fully investigated.

AIM

To evaluate the diagnostic accuracy of alpha-fetoprotein (AFP), the *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3), and protein induced by vitamin K absence or antagonist-II (PIVKA-II) and their combination for HCC diagnosis.

METHODS

Patients with newly detected liver mass or elevated serum AFP levels were considered eligible. Serum AFP level, AFP-L3 fraction, and PIVKA-II level were measured at the first visit.

RESULTS

In total, 622 patients were included; 355 patients (57.1%) had chronic liver disease, and 208 (33.4%) had liver cirrhosis. HCC was diagnosed in 160 patients (25.7%). The area under the receiver operating characteristics curves (AUROCs) of the serum AFP, AFP-L3 fraction, AFP-L3, and PIVKA-II levels for the diagnosis of HCC were 0.775, 0.792, 0.814, and 0.834, respectively. A novel diagnostic model was developed by classifying patients in a 1:1 ratio into training and validation sets. Using the binary regression analysis of the training cohort, the AFP, AFP-L3 fraction, and PIVKA-II (ALPs) score was calculated as follows: ALPs score = 3.8 × [serum AFP level (ng/mL) × AFP-L3 fraction (%) × 0.01] + 0.2 × PIVKA-II level (mAU/mL). The AUROC of the ALPs score for diagnosis of HCC was 0.878,

requirement for informed consent was waived owing to the retrospective nature of the study.

Conflict-of-interest statement: Authors have nothing to disclose.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at drseo@korea.ac.kr. Consent was not obtained but the presented data are anonymized and risk of identification is low.

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significantly higher than that of serum AFP level ($P < 0.001$), AFP-L3 fraction ($P < 0.001$), PIVKA-II level ($P = 0.036$), and AFP-L3 level ($P = 0.006$). The optimal ALPs score cut-off was 5.3 (sensitivity, 85.0%, specificity 80.1%). The validation cohort showed similar results.

CONCLUSION

The ALPs score calculated using serum AFP level, AFP-L3 fraction, and PIVKA-II level showed improved accuracy in HCC diagnosis.

Key Words: Alpha-fetoprotein; *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein; Protein induced by vitamin K absence or antagonist-II; Hepatocellular carcinoma

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Core Tip: The value of *Lens culinaris* agglutinin-reactive fraction of alpha-fetoprotein (AFP-L3) for improving diagnostic accuracy of hepatocellular carcinoma has not been fully evaluated. We investigated performance of AFP-L3 in patients with newly detected liver mass or elevated serum AFP level. In total of 622 patients, we observed significantly higher diagnostic accuracy of AFP-L3 level than with AFP level ($P < 0.001$). In addition, we developed a novel diagnostic model, AFP, AFP-L3 fraction, and protein induced by vitamin K absence or antagonist-II (PIVKA-II) score, derived from AFP level, AFP-L3 fraction, and PIVKA-II level, and it showed significantly higher area under the receiver operating characteristics curves of 0.878 than those of AFP level, AFP-L3 fraction, and PIVKA-II level (all $P < 0.05$).

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common tumor and the fourth significant cause of cancer-related death worldwide[1]. Although there have been many advancements in diagnosis of HCC, still, considerable number of patients are diagnosed as intermediate or advanced-stage HCC[2]. Therefore, improved diagnostics, especially for early-stage HCC where curative treatment is conceivable are warranted.

Imaging study plays a critical role in diagnosis of HCC, although limitations exist[3,4]. Furthermore, the imaging methods are not always applicable for certain patients, since the selection of imaging modality and contrast agent is subject to multiple factors, such as modality availability, scan time, throughput, technical capability of each institution, costs, radiologist expertise, patient preference, and safety considerations.

Therefore, although serum biomarkers usually have a minor role in the diagnosis of HCC, identifying biomarkers to better diagnose or predict HCC remains important priority in clinical research[5-7]. The American Association for the Study of Liver Diseases guideline suggests that alpha-fetoprotein (AFP) > 20 ng/mL and normal ultrasonography findings should alert recall procedures for possible HCC diagnosis, however, the routine use of novel biomarkers for HCC screening requires further evaluation[7-9]. The European association for the study of the liver guideline also indicate a lack of tumor markers for accurate and early detection of HCC owing to suboptimal previous data[5,9-11].

Various tumor markers including protein induced by vitamin K absence or antagonist-II (PIVKA-II), and novel blood parameter, the *Lens culinaris* agglutinin-reactive fraction of AFP (AFP-L3), have received considerable attention and were

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evaluated in several studies[12,13]. AFP-L3 is AFP-isoform reflecting changes in the carbohydrate chain. Since AFP-L3 showed high specificity (92.0%–99.4%) and low sensitivity (18.8%–37.0%) for HCC[14-16], it is considered a more specific biomarker for HCC diagnosis. Recent studies evaluated the predictive performance of the combination of AFP, AFP-L3, and PIVKA-II, which showed improved HCC diagnostic ability than either AFP or PIVKA-II[17,18]. However, those results were inconclusive because of the small sample size and the specific characteristics of study population.

Hence, we conducted this retrospective study with a large number of patients, who were referred to our tertiary clinic for newly detected liver mass or elevated serum AFP level. We investigated and compared the predictive ability of AFP, AFP-L3, and PIVKA-II as well as their combination for HCC diagnosis and developed and validated a new diagnostic model for HCC.

MATERIALS AND METHODS

Subjects

Patients who were referred from the primary clinic to the Korea University Medical Center (a tertiary academic teaching hospital) for newly detected liver mass or elevated serum AFP level between 2015 and 2019 were enrolled (Supplementary Figure 1). The exclusion criteria were as follows: (1) Age < 18 years; (2) Insufficient laboratory findings or imaging data; (3) History of HCC; (4) Patients with liver failure; (5) History of liver transplantation or liver resection for reasons other than HCC; and (6) History of any malignancy other than HCC. This study protocol followed the ethical guidelines of the 1975 Declaration of Helsinki, and the institutional review board of Korea University Anam Medical Center. The requirement for informed consent was waived owing to the retrospective nature of the study.

Diagnosis of HCC

HCC diagnosis was based on non-invasive criteria or pathology or both in cirrhotic patients and pathology in non-cirrhotic patients. The non-invasive criteria were based on identification of typical hallmarks of HCC, obtained by multiphasic computed tomography or multiphase magnetic resonance imaging (nodule > 1 cm with arterial hypervascularity and portal/delayed-phase washout)[19,20]. Liver cirrhosis was diagnosed when typical ultrasonographic findings were found, with a low platelet count (< 100000/μL), varices, or overt complication of cirrhosis[21,22].

Measurements of tumor markers

Serum AFP level, AFP-L3 fraction, and PIVKA-II level were measured in patient blood samples collected at their first visit. We measured the AFP level and AFP-L3 fraction using the mTAS assay (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and the PIVKA-II level using an enzyme immunoassay (Fujirebio Inc., Tokyo, Japan). Assay sensitivities were 0.3 ng/mL for AFP and 0.1 ng/mL for PIVKA-II. The percentage of AFP-L3 was determined in samples where both subfractions (AFP-L1 and AFP-L3) were > 0.3 ng/mL. The AFP-L3 level was calculated as follows: Serum AFP-L3 level = serum AFP level (ng/mL) × serum AFP-L3 fraction (%) × 0.01.

Statistical analysis

Data are presented as mean ± SD or numbers with percentages. The statistical significance of differences between continuous and categorical variables were compared using Student's *t*-test or Mann-Whitney *U* test and chi-square test, respectively. The predictive accuracies for the diagnosis of HCC were assessed using area under the receiver operating characteristics curves (AUROCs) as well as 95% confidence intervals. Optimal cutoff values were chosen to maximize the sum of the sensitivity and specificity. The sensitivity, specificity, positive predictive value (PPVs), and negative predictive value (NPVs) were computed for the optimal cutoff value. The AUROCs were compared using the Hanley-McNeil test[23]. Statistical analyses were conducted using IBM SPSS Statistics software (version 23.0.0.0., IBM Corporation, Armonk, NY, United States). Two-sided *P* values < 0.05 were considered significant.

RESULTS

Baseline characteristics of the study population

In total, 622 patients were finally included for the statistical analysis. The baseline characteristics of the study population are presented in Table 1**.** In the study population, chronic liver disease and liver cirrhosis were present in 355 (57.1%) and 208 (33.4%) patients, respectively. Among the 160 (25.7%) patients with HCC, BCLC stage was 0 in 35 (21.9%) patients, A in 50 (31.3%) patients, B in 32 (20.0%) patients, C (26.3%) in 42 patients, and D (0.6%) in 1 patients. The mean serum AFP, fraction of AFP-L3, AFP-L3 level and PIVKA-II level were significantly higher in patients with HCC than in those without HCC (all $P < 0.001$) (Table 1).

Diagnostic accuracy of tumor markers for HCC

For HCC diagnosis, AFP level, fraction of AFP-L3, level of AFP-L3 and PIVKA-II level had AUROC values of 0.775, 0.792, 0.814, and 0.834, respectively (Figure 1). The AUROC of the serum AFP level was similar to that of serum AFP-L3 fraction ($P = 0.321$), though significantly lower than that of the serum AFP-L3 level ($P < 0.001$) and PIVKA-II level ($P = 0.038$). The AUROCs of the serum AFP-L3 fraction, AFP-L3 level, and PIVKA-II level were similar (Figure 1). For diagnosis of HCC, The optimal cutoff values of the AFP level, fraction of AFP-L3, level of AFP-L3, and PIVKA-II level were 4.2 ng/mL, 5.7%, 0.3 ng/mL, and 35.0 mAU/mL, respectively.

Development of a novel diagnostic model

A novel HCC diagnostic model was developed by classifying all included patients into training and validation sets in a 1:1 ratio under stratifying them by presence or absence of HCC. The baseline characteristics did not differ significantly between the two datasets (Supplementary Table 1).

In the training set, the mean serum AFP, fraction of AFP-L3, AFP-L3 level and PIVKA-II level were significantly higher in patients with HCC than in those without HCC (all $P < 0.001$). The AUROCs of serum AFP level, AFP-L3 fraction, PIVKA-II level and AFP-L3 levels were 0.749, 0.786, 0.829, and 0.802, respectively. The AUROC of the serum AFP-L3 level was significantly higher than that of serum AFP level ($P < 0.001$), whereas no significant difference was observed in the other tumor marker comparisons (all $P > 0.05$).

Next, we performed binary regression analysis for the presence of HCC using the levels of two tumor markers, AFP-L3 level and PIVKA-II level (Table 2). Using the beta coefficients of each marker, we developed AFP, fraction of AFP-L3, and PIVKA-II (ALPs) score: ALPs score = 3.8 × [serum AFP level (ng/mL) × fraction of AFP-L3 (%) × 0.01] + 0.2 × PIVKA-II level (mAU/mL). In training set, mean ALPs score was significantly higher in patients with HCC than in those without HCC (10657.4 *vs* 11.9, $P < 0.001$). For the HCC diagnosis, ALPs score had significantly higher AUROC of 0.878, than those of serum AFP level ($P < 0.001$), fraction of AFP-L3 ($P < 0.001$), level of PIVKA-II ($P = 0.036$), and level of AFP-L3 ($P = 0.006$) (Figure 2A). The best cutoff value of ALPs score was 5.3 (sensitivity, 85.0%, specificity 80.1%, PPV 59.6%, and NPV 93.9%).

In the validation set, the mean serum AFP, fraction of AFP-L3, AFP-L3 level and PIVKA-II level were significantly higher in patients with HCC than in those without HCC (all $P < 0.001$). With an optimal ALPs score cutoff value of 5.3, the sensitivity, specificity, PPV, and NPV were 85.0%, 77.5%, 56.7%, and 93.7%, respectively. The AUROCs of the serum AFP level, AFP-L3 fraction, PIVKA-II level, AFP-L3 level, and ALPs score were 0.800, 0.798, 0.839, 0.825, and 0.897, respectively. The AUROC of serum AFP-L3 level was also significantly higher than that of serum AFP level ($P > 0.001$), whereas the ALPs score had higher AUROC than those of level of AFP ($P < 0.001$), fraction of AFP-L3 ($P < 0.001$), level of PIVKA-II ($P = 0.024$), and level of AFP-L3 ($P = 0.002$) (Figure 2B).

Diagnostic accuracy of the ALPs score and other tumor markers for very early and early-stage HCC

In total, 53 (8.5%) patients had very early and early-stage HCC. The baseline characteristic of these patients are presented in Supplementary Table 2. The predictive performance of the ALPs score and other tumor markers for very early and early-stage HCC was evaluated in all patients. The AUROCs of the level of AFP, fraction of AFP-L3, level of AFP-L3, level of PIVKA-II and ALP score for diagnosing very early and early-stage HCC were 0.631, 0.670, 0.679, 0.683, and 0.746, respectively. The AUROC of the serum AFP-L3 level was significantly higher than that of the serum AFP level ($P <$

Table 1 Baseline characteristics of the study population according to the presence of hepatocellular carcinoma

Variables	All patients (*n* = 622)	Patients without HCC (*n* = 462)	Patients with HCC (*n* = 160)	*P* value
Demographic variables				
Age, years	56.5 ± 13.5	54.1 ± 13.4	63.5 ± 11.3	< 0.001
Male gender	344 (55.3)	226 (48.9)	118 (73.8)	< 0.001
Chronic liver disease	355 (57.1)	198 (42.9)	157 (98.1)	< 0.001
Etiology of liver disease				< 0.001
Hepatitis B virus	211 (33.9)	114 (24.7)	97 (60.6)	
Hepatitis C virus	26 (4.2)	7 (1.5)	19 (11.9)	
Alcoholic liver disease	72 (11.6)	39 (8.4)	33 (20.6)	
Others	46 (7.4)	38 (8.2)	8 (5.0)	
Liver cirrhosis	208 (33.4)	91 (19.7)	117 (73.1)	< 0.001
Laboratory variables				
Total bilirubin, mg/dL	1.4 ± 2.7	1.3 ± 2.7	1.8 ± 2.7	0.072
Albumin, g/dL	4.2 ± 1.7	4.3 ± 1.9	3.8 ± 0.7	< 0.001
Prothrombin time, INR	1.1 ± 0.2	1.0 ± 0.2	1.1 ± 0.2	< 0.001
Alanine aminotransferase, IU/L	44.8 ± 88.3	43.8 ± 99.6	47.8 ± 42.1	0.624
AFP level, ng/mL	3638.6 ± 58824.2	18.2 ± 63.8	14092.6 ± 115614.1	< 0.001
AFP-L3 fraction, %	6.7 ± 16.7	1.9 ± 3.9	20.6 ± 28.0	< 0.001
AFP-L3 level, ng/mL	833.1 ± 8811.3	1.1 ± 4.7	3235.3 ± 17185.1	< 0.001
PIVKA-II level, mAU/mL	1613.4 ± 10561.6	37.9 ± 257.3	6162.5 ± 20185.1	< 0.001
ALPs score	3488.4 ± 33695.4	11.9 ± 54.4	13526.8 ± 65558.1	< 0.001

Variables are expressed as mean ± SD or *n* (%). HCC: Hepatocellular carcinoma; AFP: Alpha-fetoprotein; AFP-L3: *Lens culinaris* agglutinin- reactive fraction of AFP; PIVKA-II: Protein induced by vitamin K absence or antagonist-II; ALPs: AFP, AFP-L3 fraction, and PIVKA-II.

Table 2 Binary regression analysis for the presence of hepatocellular carcinoma

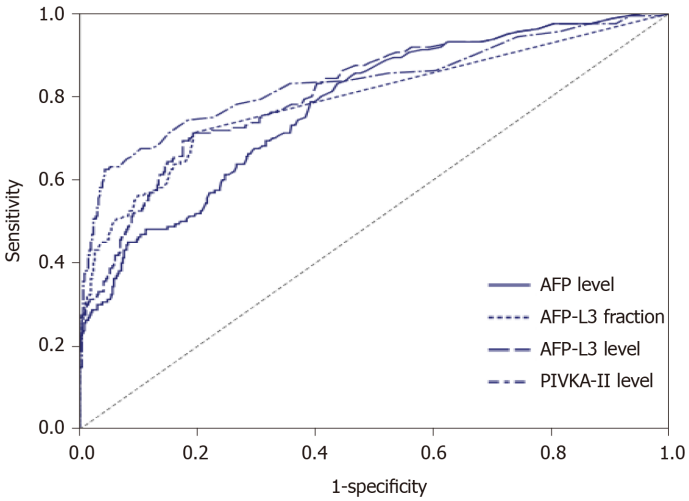
	Beta coefficient	Standard error	*P* value
AFP-L3 level	0.038	0.017	0.021
PIVKA-II level	0.002	0.001	0.003

HCC: Hepatocellular carcinoma; AFP: Alpha-fetoprotein; PIVKA-II: Protein induced by vitamin K absence or antagonist-II.

0.001), whereas the ALPs score had higher AUROC than those of level of AFP (P < 0.001), fraction of AFP-L3 (P = 0.009), level of PIVKA-II (P = 0.015), and level of AFP-L3 (P = 0.010) (Figure 3). The optimal cutoff value of ALPs score was 6.7 (sensitivity 70.6%, specificity 85.5%, PPV 47.2%, and NPV 94.5%).

DISCUSSION

The ability of AFP-L3 alone or its combination with other markers for diagnosis of HCC has not been fully evaluated. In this large retrospective study of patients with newly detected liver mass or elevated serum AFP level, we observed a significantly higher diagnostic accuracy of AFP-L3 level than with AFP level (P < 0.001). In addition, we developed a novel diagnostic model for HCC, ALPs score, which is derived from the AFP level, AFP-L3 fraction, and PIVKA-II level. For HCC diagnosis, ALPs score had AUROC of 0.878, which was significantly higher than those of the AFP



Variables	AUROC (95%CI)	Cut-off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
AFP level	0.775 (0.740-0.807)	4.2 ng/mL	78.8	60.8	41.0	89.2
AFP-L3 fraction	0.792 (0.758-0.823)	5.7%	71.3	80.7	56.2	89.0
AFP-L3 level	0.814 (0.781-0.844)	0.3 ng/mL	71.3	80.7	56.2	88.8
PIVKA-II level	0.834 (0.802-0.862)	35.0 mAU/mL	62.5	95.7	83.3	88.0

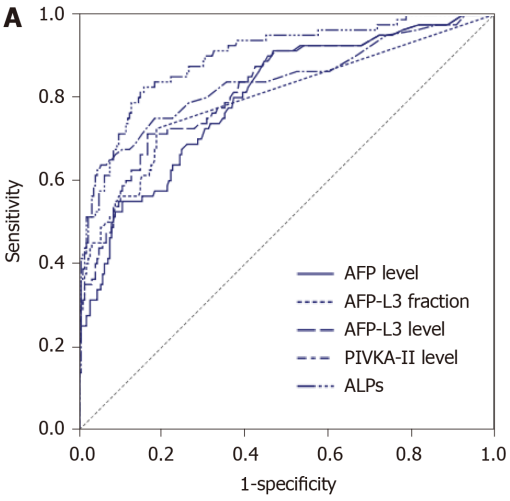
Figure 1 The area under the receiver operating characteristic curves of the tumor markers for hepatocellular carcinoma diagnosis in the total population. AUROC: Area under the receiver operating characteristic curve; HCC: Hepatocellular carcinoma; AFP: Alpha-fetoprotein; AFP-L3: *Lens culinaris* agglutinin-reactive fraction of AFP; PIVKA-II: Protein induced by vitamin K absence or antagonist-II; NPV: Negative predictive value; PPV: Positive predictive value.

level, fraction of AFP-L3, and PIVKA-II level (all $P < 0.05$).

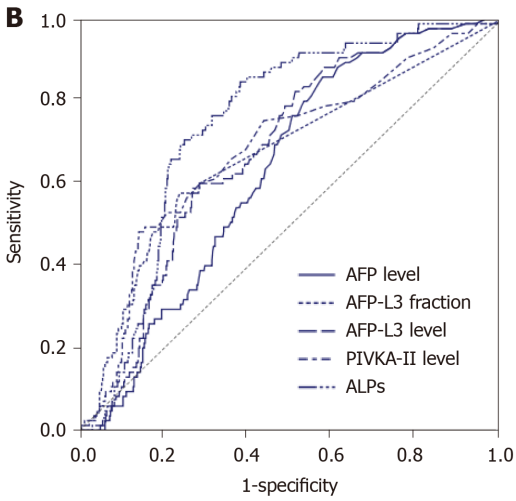
Our study has several strengths and clinical implications. First, we showed the significantly higher diagnostic accuracy of the AFP-L3 level in comparison with the AFP level for HCC diagnosis ($P < 0.001$). The AUROC of the serum AFP-L3 level was 0.814, and the optimal cutoff was 0.3 ng/mL with sensitivity of 71.3% and specificity of 80.7%. Although the AFP-L3 has been expected to compensate for low specificity of AFP level, previous studies showed conflicting results regarding the superiority of AFP-L3 over AFP for diagnosis of HCC[17,18,24,25]. However, in previous studies, the AFP-L3 fraction, and not the AFP-L3 level, was compared to the AFP level, thus leading to comparable results between the two variables. The present study also showed the comparable diagnostic accuracy between AFP-L3 fraction and the AFP level for HCC diagnosis ($P = 0.321$); however, the AFP-L3 level, calculated by multiplying the AFP level with the AFP-L3 fraction, showed consistent superiority over the AFP level for HCC diagnosis. Therefore, the use of AFP-L3 level rather than AFP-L3 fraction might be beneficial in future studies.

Second, we developed and validated a novel diagnostic model of HCC, ALPs score, derived from the combination of the AFP and PIVKA-II levels, and AFP-L3 fraction. The AUROC of the ALPs score for HCC diagnosis was 0.878, which was superior than those of the level of AFP, fraction of AFP-L3, AFP-L3 level, and PIVKA-II level (all $P < 0.05$) in both the training and validation sets. The optimal ALPs score cutoff for HCC diagnosis was 5.3 with sensitivity of 85.0% and specificity of 77.5%–80.1%. It seems that the high sensitivity and specificity of ALPs score, which could be easily obtained from simple blood tests has significant clinical implication. Lim *et al*[18] showed that the combined use of all three tumor markers improved the diagnostic accuracy compared to each marker alone in detecting HCC (AUROC of 0.877) and early-stage HCC (AUROC of 0.773). However, the specificity of the combination of three tumor markers for HCC diagnosis in Lim *et al*[18] was much lower than that of present study (60.1% *vs* 77.5%–80.1%), and this difference might be caused from the different patient population between two studies. In addition, our study derives its strength compared to the study by Lim *et al*[18] by developing a novel score with the diagnostic value of each marker, which was reweighted through binary regression analysis.

Recently, Best *et al*[17] introduced the GALAD score, derived from a combination of gender, age, AFP level, AFP-L3 fraction, and PIVKA-II level with an AUROC of 0.9242 (sensitivity of 85.6% and specificity of 93.3%), for detecting (very) early-stage HCC. However, such high AUROC for (very) early-stage HCC with only serum markers seems clinically unacceptable. In this study, the AUROC of ALPs score to predict very



Variables	AUROC (95%CI)
AFP level	0.800 (0.751-0.843)
AFP-L3 fraction	0.798 (0.750-0.841)
AFP-L3 level	0.839 (0.793-0.878)
PIVKA-II level	0.825 (0.778-0.866)
ALPs score	0.897 (0.857-0.928)



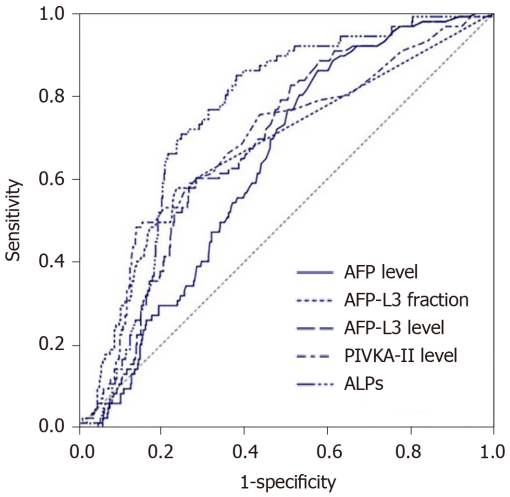
Variables	AUROC (95%CI)
AFP level	0.697 (0.643-0.751)
AFP-L3 fraction	0.728 (0.660-0.795)
AFP-L3 level	0.752 (0.698-0.806)
PIVKA-II level	0.756 (0.692-0.821)
ALPs score	0.836 (0.789-0.883)

Figure 2 The area under the receiver operating characteristic curves of tumor markers for hepatocellular carcinoma diagnosis in the training set and the validation set. A: Training set; B: Validation set. AUROC: Area under the receiver operating characteristic curve; HCC: Hepatocellular carcinoma; AFP: Alpha-fetoprotein; AFP-L3: *Lens culinaris* agglutinin-reactive fraction of AFP; PIVKA-II: Protein induced by vitamin K absence or antagonist-II; ALPs: AFP, AFP-L3 fraction, and PIVKA-II.

early and early-stage HCC was 0.746, which was significantly superior compared to other markers (all $P < 0.05$). Owing to the small number of patients with very early and early-stage HCC in our study (53 patients), we were unable to confirm the clinical validity of the ALPs score for the prediction of very early and early-stage HCC. Large multicenter cohort studies are required to evaluate the predictive ability of the ALPs score for diagnosing very early and early-stage HCC.

The use of AFP as a diagnostic marker for HCC has some shortcomings, including an increase in diseases other than HCC[26], large false-negatives, and missed diagnoses[27]. Although PIVKA-II is less likely to be elevated in other liver disease than AFP[26], the results of previous studies were restrictive to prove the sufficient role of PIVKA-II as a solitary biomarker for HCC diagnosis, since the superior ability of PIVKA-II over AFP was only shown in specific patient groups[14,28,29]. Therefore, recent studies investigated the ability of combined AFP and PIVKA-II in HCC diagnosis and showed the higher diagnostic ability of the combination of the two markers compared to that of either biomarker alone[10,30]. AFP and PIVKA-II complement each other's role, and similarly, AFP-L3 could act as a complementary biomarker for AFP and PIVKA-II in diagnosing HCC while improving the sensitivity and specificity.

We are also aware of several issues that remain unresolved. First, since this was a retrospective study, potential selection bias might be present. Second, 42.9% of the enrolled patients had no underlying liver disease, hence, the clinical implications of the ALPs score in patients with chronic liver disease could not be evaluated. However, because many patients without any underlying liver disease also have elevated AFP level or liver nodule, the result of our study could be beneficial in clinical practice. Further studies including patients with specific liver disease and studies for external validation are required. Third, the cost of using combination of tumor markers is another issue, therefore, further studies evaluating the cost-effectiveness are warranted.



Variables	AUROC (95%CI)
AFP level	0.631 (0.578-0.684)
AFP-L3 fraction	0.670 (0.605-0.736)
AFP-L3 level	0.679 (0.626-0.733)
PIVKA-II level	0.683 (0.621-0.746)
ALPs score	0.746 (0.698-0.794)

Figure 3 The area under the receiver operating characteristic curves of tumor markers for the diagnosis of very early and early-stage hepatocellular carcinom in the total population. AUROC: Area under the receiver operating characteristic curve; HCC: Hepatocellular carcinoma; AFP: Alpha-fetoprotein; AFP-L3: *Lens culinaris* agglutinin-reactive fraction of AFP; PIVKA-II: Protein induced by vitamin K absence or antagonist-II; ALPs: AFP, AFP-L3 fraction, and PIVKA-II.

CONCLUSION

In conclusion, AFP-L3 level, calculated by multiplying the AFP level with the AFP-L3 fraction, exhibited a considerably higher diagnostic ability than the AFP level for HCC diagnosis. The novel diagnostic model, ALPs score may be valuable compared to individual tumor markers for HCC diagnosis in patients with and without chronic liver disease.

ARTICLE HIGHLIGHTS

Research background

Identifying useful biomarkers to diagnose hepatocellular carcinoma (HCC) remains important priority in clinical research. Recently, various tumor markers including protein induced by vitamin K absence or antagonist-II (PIVKA-II), and novel blood parameter, the *Lens culinaris* agglutinin- reactive fraction of alpha-fetoprotein (AFP-L3), have been introduced and evaluated.

Research motivation

Several studies showed improved HCC diagnostic ability of the combination of AFP, AFP-L3, and PIVKA-II, than either AFP or PIVKA-II. However, because of the small sample size and the specific characteristics of the study population, conflicting results have been reported.

Research objectives

We aimed to investigate and compare the predictive ability of AFP, AFP-L3, and PIVKA-II as well as their combination for HCC diagnosis in this retrospective study with a large number of patients. Further, we developed and validated a new diagnostic model for HCC.

Research methods

Patients referred from primary clinics to the Korea University Medical Center for newly noted space occupying lesion in liver or elevated serum AFP level between 2015 and 2019 were enrolled. Their serum AFP level, AFP-L3 fraction, and PIVKA-II level were measured in blood samples collected at their first visit.

Research results

It was observed that AFP-L3 level exhibited a considerably higher diagnostic accuracy than with AFP level ($P < 0.001$). In addition, a novel diagnostic model for HCC, ALPs score, derived from the AFP level, AFP-L3 fraction, and PIVKA-II level showed highest area under the receiver operating characteristics curves of 0.878 for HCC diagnosis. It was significantly higher than those of the AFP level, AFP-L3 fraction, and PIVKA-II level (all $P < 0.05$).

Research conclusions

Finally, AFP-L3 level, calculated by multiplying the AFP level with the AFP-L3 fraction had a significantly higher diagnostic ability than the AFP level for HCC diagnosis. The novel diagnostic model, ALPs score was more valuable compared to individual tumor markers for HCC diagnosis.

Research perspectives

Further studies including patients with specific liver disease and studies for external validation and cost-effectiveness are required.

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ORIGINAL ARTICLE

Retrospective Study

New anti-reflux plastic stent to reduce the risk of stent-related cholangitis in the treatment of biliary strictures

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Author contributions: Hu B, Yuan XL, and Ye LS designed this study; Yuan XL, Ye LS, and Zeng XH analyzed and interpreted the data; Yuan XL, Ye LS, and Zeng XH drafted the manuscript; Hu B, Tan QH, Mou Y, Liu W, Wu CC, and Yang H critically revised the manuscript.

Institutional review board statement: The study protocol was approved by the Ethics Committee on Biomedical Research, West China Hospital of Sichuan University.

Informed consent statement: Informed consent was waived by the Ethics Committee because of the retrospective nature of this study and anonymous data.

Conflict-of-interest statement: Prof. Bing Hu is one of the inventors of the antireflux plastic stent. He has worked in collaboration with

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Abstract

BACKGROUND

There is little data available on the role of new anti-reflux plastic stents (ARPSs).

AIM

To compare the use of ARPSs with that of traditional plastic stents (TPSs) for patients with biliary strictures.

METHODS

Consecutive patients with biliary strictures who underwent first endoscopic biliary stenting between February 2016 and May 2019 were included. The onset of stent-related cholangitis, stent patency, clinical success, and other adverse events were evaluated.

RESULTS

Sixty-seven patients in the ARPS group and 66 patients in the TPS group were included in the final analyses. Fewer patients experienced stent-related cholangitis in the ARPS group than that in the TPS group (8 patients *vs* 18 patients; $P = 0.030$). The median time till the onset of first stent-related cholangitis was later in the ARPS group than that in the TPS group (128.5 d *vs* 76 d; $P = 0.039$). The cumulative median stent patency in the ARPS group was 185 d, which was significantly longer than that in the TPS group (133 d; $P = 0.001$). The clinical success rates and other adverse events did not significantly differ between both groups.

CONCLUSION

Placement of new ARPS might be a safe and effective optional therapeutic strategy to reduce the risk of stent-related cholangitis and prolong stent patency.

Micro-Tech (Nanjing) Co. Ltd., Nanjing, China, to develop the stent. No free device was offered for this study. Authors disclosed no conflicts of interests.

Data sharing statement: No additional unpublished data are available.

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Core Tip: This is a retrospective study to compare the use of new anti-reflux plastic stents (ARPSs) with that of traditional plastic stents (TPSs) for patients with biliary strictures. Fewer patients experienced stent-related cholangitis in the ARPS group than in the TPS group (8 *vs* 18 patients; $P = 0.030$). The cumulative median stent patency in the ARPS group was 185 d, which was significantly longer than that in the TPS group (133 d).

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INTRODUCTION

Endoscopic biliary stenting is an established procedure for either benign or malignant biliary strictures (BBSs or MBSs) that are not suitable for surgery. There are two stent categories available on the market: Plastic and metal stents. Plastic stents (PSs) are widely available, very effective, and inexpensive. PSs with a diameter of 10 Fr are usually used. However, the rather short stent patency (approximately 3 to 6 mo)[1] and the relatively high rate of stent-related cholangitis (about 3.5%-48.8%)[2-4] represent significant disadvantages. In contrast, self-expandable metal stents (SEMSs) are associated with significantly longer stent patency (approximately 9 to 12 mo)[5] compared to that of PSs. The rate of stent-related cholangitis, however, can reach as high as 45.8% (about 2.1%-45.8%)[3,6]. Most importantly, the higher costs of SEMSs limit their widespread application, especially in developing countries[7].

Both stent categories have complications of stent occlusion and stent-related cholangitis after stent placement, which may worsen the quality of patients' life. Since duodenobiliary reflux has been identified as a major cause of these problems[1,8-10], the design of biliary stents with an anti-reflux valve at the duodenal end has emerged as a potential solution for reducing duodenobiliary reflux over recent years. Previous studies[10-12] have shown that the use of anti-reflux SEMSs can effectively reduce the risk of stent-related cholangitis and prolong stent patency. Unfortunately, studies on anti-reflux PSs (ARPSs) have not yet yielded convincing results[7,13,14].

We designed an ARPS with a "duckbilled" valve attached to the PS. In the prior clinical trial, 19 patients with distal MBSs were successfully inserted with the new ARPSs, and the cumulative median stent patency of the ARPSs was significantly longer than that of traditional PSs (TPSs)[15]. However, the role of this new ARPS in reducing the onset of stent-related cholangitis compared with TPS remains unknown. In the present study, we aimed to determine whether placement of this new ARPS can reduce the risk of stent-related cholangitis in patients with biliary strictures compared to TPS.

MATERIALS AND METHODS

Study design

Consecutive patients with biliary strictures who underwent first endoscopic biliary stenting between February 2016 and May 2019 at West China Hospital, Sichuan University were included. We retrospectively reviewed the data of eligible patients from our prospectively collected database. Exclusion criteria were (1) Placement of SEMS; (2) Placement of multiple biliary stents; (3) Stents with a diameter other than 10Fr; (4) Simultaneous percutaneous transhepatic cholangial drainage and endoscopic

biliary stenting; (5) Temporary drainage for resectable MBS; (6) Patients died within 1 mo after stenting; and (7) Patients without available outcome data. Patient characteristics, endoscopic retrograde cholangiopancreatography (ERCP) procedures, stent details, liver function test results, adverse events, and stent patency duration were collected.

This study was approved by the Ethics Committee on Biomedical Research, West China Hospital of Sichuan University. Informed consent was waived by the Ethics Committee because of the retrospective nature of this study and anonymous data.

Plastic stents

The new ARPS (Tannenbaum design; Micro-Tech (Nanjing) Co. Ltd., Nanjing, China) with a diameter of 10 Fr was made of polytetrafluoroethylene, without any side holes (Figure 1A). A soft anti-reflux valve with a length of 1.5 cm, made of silicone rubber material, was attached to the PS. The antegrade flow of bile kept the valve open, whereas increased intestinal pressure made it close, thereby reducing duodenobiliary reflux. The TPS (Cotton-Leung Biliary Stent; Cook Ireland Ltd., Limerick, Ireland) used in this study was made of polyethylene (Figure 1B). Both types of stents were inserted into the bile duct using the same pull-back delivery system (Oasis; Cook Ireland Ltd.).

Endoscopic biliary stenting

Except for patients who were randomly assigned during the previous study period [15], other patients were advised to choose ARPSs during preoperative preparation, and TPSs were used for those who refused ARPSs. We did not administer prophylactic antibiotics and nonsteroidal anti-inflammatory drugs prior to ERCP. All ERCPs were performed by experienced endoscopists (experience of ≥ 300 ERCPs per year) using a standard duodenoscope (TJF-260V; Olympus Medical systems Corp., Tokyo, Japan). All patients were placed in the prone position with conscious sedation. After bile duct cannulation, the stricture was visualized by injecting contrast. A sphincterotomy was performed if necessary. Under the guidance of a guidewire (Jagwire; Boston Scientific, Natick, MA. United States), a single ARPS or TPS with an individually determined ideal length was inserted into the bile duct above the proximal end of the stricture for about 1-2 cm, and the distal end of the stent was kept outside of the duodenal papilla for about 1cm. The flow of bile was confirmed before the withdrawal of the duodenoscope (Figure 2). Patients received clinical evaluation and liver function tests within the first week after stent placement. Then, they underwent clinic visits or telephone follow-up every 1-2 mo until stent removal/exchange or death. The follow-up ended in December 2019.

Outcomes

The primary outcome was the onset of stent-related cholangitis. Stent-related cholangitis, including non-stent dysfunction cholangitis and stent dysfunction cholangitis, was considered if patients developed symptoms of acute cholangitis during the follow-up period and post-ERCP cholangitis was excluded. Non dysfunction cholangitis was diagnosed when acute cholangitis observed and when there was no definite finding of stent occlusion or migration[16]. Post-ERCP cholangitis was defined as cholangitis that developed within 2 wk after ERCPs[17] without definite findings of stent dysfunction, related to ERCP procedures,. The diagnostic criteria for acute cholangitis refer to Tokyo Guidelines 2018[18]. Stent dysfunction was confirmed by subsequent ERCP findings when patients presented with recurrent obstructive jaundice and/or cholangitis symptoms in combination with resolution of these abnormalities after insertion of a new stent.

Secondary outcomes were stent patency, clinical success, and other adverse events. The duration of stent patency was measured as the days from stent insertion until stent dysfunction requiring stent exchange. Clinical success was defined as a decrease in bilirubin levels of at least 50% within the first week after stent placement in patients with jaundice[19]. Other adverse events were defined according to American Society for Gastrointestinal Endoscopy guidelines[20], such as post-ERCP pancreatitis and stent migration. Stent migration included proximal and distal migration. Proximal migration was defined if the stent was visible in bile duct on fluoroscopy, but the distal end of the stent was not seen at duodenal papilla on endoscopy[21]. Partial distal migration was defined if the stent location was below the original position on fluoroscopy, and the distal end of the stent was seen at duodenum on endoscopy. Complete distal migration was defined if the stent was not present on fluoroscopy and endoscopy. Proximal biliary stricture was defined as stricture located in the common hepatic duct, while distal stricture was defined as stricture located in the common bile

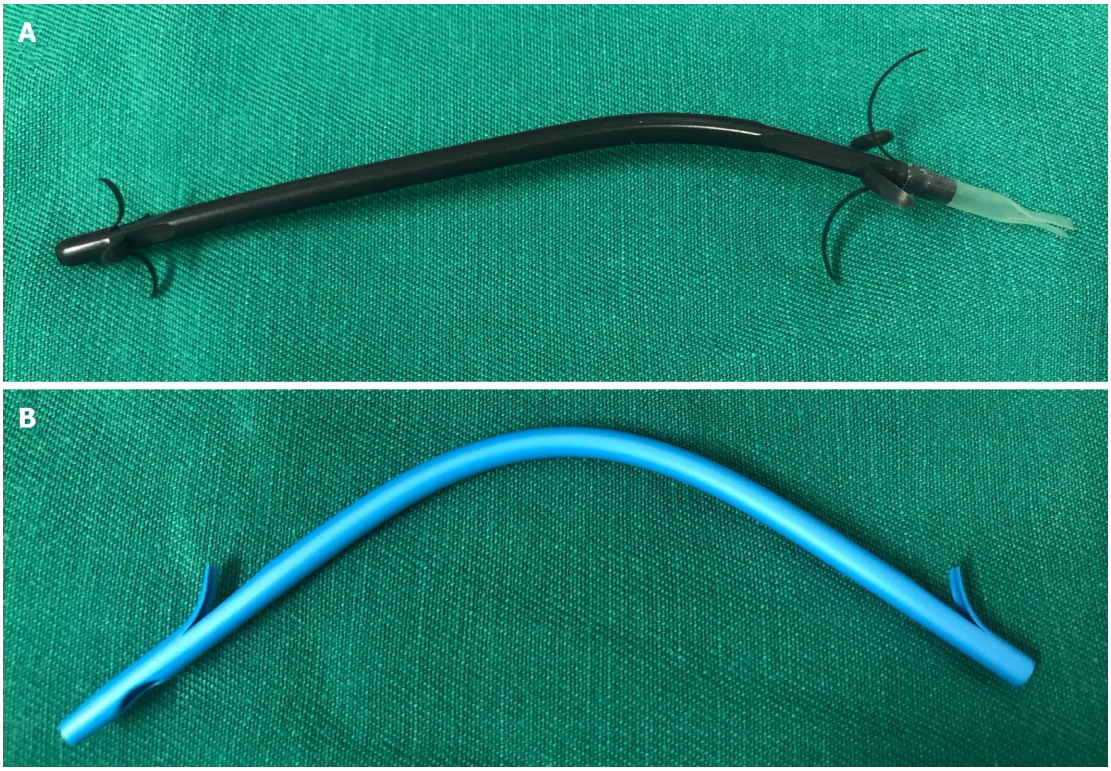


Figure 1 Biliary stents used in this study. A: The new anti-reflux plastic stent; B: The traditional plastic stent.

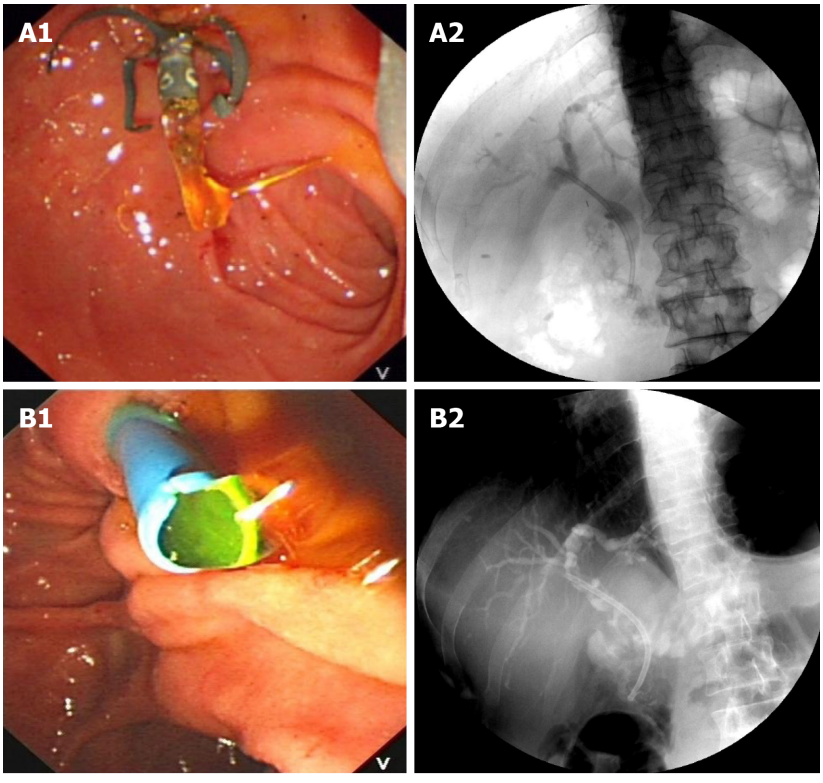


Figure 2 Successful endoscopic placement of an anti-reflux plastic stent or a traditional plastic stent. A1 and A2: Endoscopic and fluoroscopic images of an anti-reflux plastic stent; B1 and B2: Endoscopic and fluoroscopic images of a traditional plastic stent.

duct.

Statistical analysis

Continuous variables were characterized using mean with standard deviation or

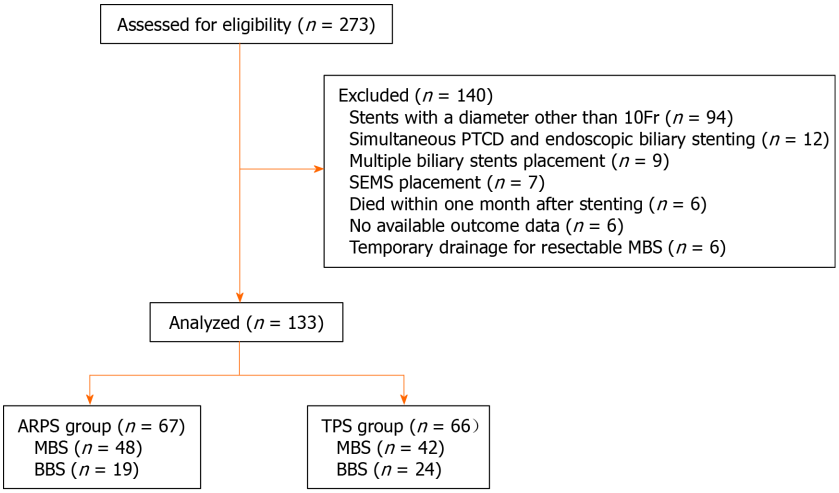


Figure 3 Flowchart. PTCD: Percutaneous transhepatic cholangial drainage; SEMS: Self-expandable metal stents; ARPS: Anti-reflux plastic stent; TPS: Traditional plastic stent. MBS: Malignant biliary stricture; BBS: Benign biliary stricture.

median with interquartile range (IQR), while categorical using frequencies or proportions. Exploratory subgroup analyses for MBS and BBS were performed. The differences of parametric variables between the two groups were compared using Student's *t* test, and nonparametric variables were compared using the Mann-Whitney *U* est. Chi-squared test or Fisher's exact test was used for comparison of differences in categorical variables as appropriate. The cumulative onset of stent-related cholangitis and stent patency of the two groups were plotted using the Kaplan-Meier method and were analyzed using Log-rank test. A P value of < 0.05 was considered statistically significant. All analyses were performed using SPSS v. 23.0 (IBM Corp., Armonk, NY, United States).

RESULTS

Patient characteristics and clinical outcomes

From February 2016 to May 2019, a total of 274 eligible patients who underwent first endoscopic biliary stenting were screened. Among these, 140 patients were excluded due to the presence of exclusion criteria; thus, 133 patients were included in the final analyses, including 67 patients in the ARPS group and 66 patients in the TPS group (Figure 3).

The baseline demographic data, liver function test results, cholecystectomy history, and comorbidities did not significantly differ between the two groups (Table 1). There were 90 (67.7%) patients of MBSs and 43 (32.3%) patients of BBSs in the two groups. Among patients with MBSs, 44 (48.9%) patients had pathological confirmation of malignancy through brush cytology, transpapillary biopsy, or fine needle aspiration sampling. Also, 46 (51.1%) patients with imaging-evidenced tumors were confirmed by clinical follow-up (Table 2). All ARPSs or TPSs were successful placement in an appropriate position as confirmed by fluoroscopy. Clinical success was achieved in 123 (92.5%) patients, while there was no significant difference between the ARPS (n = 62) and TPS groups (n = 61) (92.5% *vs* 92.4%; P = 1.000).

Stent-related cholangitis

During the follow-up period, 8 (11.9%) patients in the ARPS group and 18 (27.3%) patients in the TPS group developed stent-related cholangitis, and a significant difference was noted (P = 0.030). Among 8 patients with stent-related cholangitis in the ARPS group, 2 patients had pre-ERCP cholangitis, and 2 patients had non-stent dysfunction cholangitis. One patient had proximal stricture, and 7 patients had distal stricture. While 18 patients in the TPS group, 6 patients had pre-ERCP cholangitis, and 6 patients had non-stent dysfunction cholangitis. Three patients had proximal stricture, and 15 patients had distal stricture. The rates of pre-ERCP cholangitis (P = 0.657), onset of non-stent dysfunction cholangitis (P = 0.165), and distribution of stricture location (P = 1.000) did not significantly differ between both groups. In the MBS or BBS subgroup, no significant difference in the onset of stent-related cholangitis

Table 1 Detailed characteristics of patients in the anti-reflux plastic stent group and traditional plastic stent group

	ARPS group	TPS group	*P* value
Patients, *n*	67	66	-
Sex, male/female	39/28	37/29	0.802[3]
Age, yr[1]	63.4 (16.6); [24-99]	65.4 (14.7); [31-93]	0.225[4]
Etiologies			0.324[3]
MBS	48	42	
BBS	19	24	
Preoperative liver function test results[2]			
Total bilirubin, mmol/L	126.0 (196.9); [7.2-523.3]	156.1 (224.3); [6.8-536.5]	0.787[5]
Direct bilirubin, mmol/L	117.3 (163.5); [2.1-417.7]	136.1 (217.9); [2.3-456.3]	0.822[5]
Alkaline phosphatase, IU/L	401.0 (456.0); [75.0-2240.0]	408.0 (400.7); [77.0-1928.0]	0.302[5]
Glutamyl-transpeptidase, IU/L	558.0 (543.0); [25.0-2067.0]	421.0 (546.7); [19.0-2488.0]	0.091[5]
Cholecystectomy history	14 (20.9%)	22 (33.3%)	0.106[3]
Cholangitis prior to ERCP	8 (11.9%)	15 (22.7%)	0.100[3]
Cholelithiasis	11 (16.4%)	16 (24.2%)	0.262[3]
Duodenal parapapillary diverticulum	4 (6.0%)	4 (6.1%)	1.000[6]
Endoscopic sphincterotomy	42 (62.7%)	38 (57.6%)	0.547[3]
Stricture location, proximal/distal	12/55	11/55	0.850[3]
Stent length, cm[2]	7.0 (3.0); [5.0-12.0]	7.0 (4.0); [5.0-14.0]	0.692[5]

[1]Mean (standard deviation) [range].
[2]Median (interquartile range) [range].
[3]Chi-squared test.
[4]Student's *t* test.
[5]Mann-Whitney *U* test.
[6]Fisher's exact test. ARPS: Anti-reflux plastic stent; TPS: Traditional plastic stent; ERCP: Endoscopic retrograde cholangiopancreatography; MBS: Malignant biliary stricture; BBS: Benign biliary stricture.

was observed between the two groups. The median time till the onset of first stent-related cholangitis in the ARPS group was later than that in the TPS group (128.5 d, IQR 197.5, range 65-347 d *vs* 76 d, IQR 72.8, range 5-337; $P = 0.039$), and there was also a similar trend in the cumulative median time between the two groups (Figure 4). In the ARPS group, 6 patients had stent-related cholangitis only once, 2 patients had it twice. In the TPS group, 12 patients had it once, 5 patients had it twice, and 1 patient had it three times. There was no significant difference in the frequency of stent-related cholangitis between the two groups ($P = 0.115$) (Table 3).

Stent patency

Up till the final follow-up, stent dysfunction was noted in 44 (65.7%) patients in the ARPS group and 52 (78.8%) patients in the TPS group, with median stent patency of 124 d (IQR 139, range 6-474) and 114 d (IQR 76.5, range 4-353), respectively. There were no significant differences in the occurrence of stent dysfunction ($P = 0.091$) and the median stent patency between the two groups ($P = 0.135$).

In the remaining patients, stent patency was maintained until stent removal/exchange or death. The cumulative median stent patencies were 185 d (SE 8.6; 95%CI: 168.2-201.8) in the ARPS group and 133 d (SE 16.9; 95%CI: 99.8-166.2) in the TPS group; the observed difference was statistically significant ($P = 0.001$). The cumulative median stent patency was 124 d (SE 26.2; 95%CI: 72.7-175.3) in patients with stent-related cholangitis in the ARPS group, while it was 96 d (SE 22.1; 95%CI: 52.7-139.3) in the TPS group ($P = 0.420$). In the MBS subgroup, the cumulative median stent patencies in the ARPS and TPS groups were 173 d (SE 32.0; 95%CI: 110.3-235.7) and 98 d (SE 11.6; 95%CI: 75.3-120.7), respectively; the observed difference was statistically significant ($P = 0.008$). In the BBS subgroup, the cumulative median stent patency in

Table 2 Etiologies of strictures in the anti-reflux plastic stent group and traditional plastic stent group

	Etiologies	ARPS group, *n* = 67	TPS group, *n* = 66	Total, *n* = 133	*P* value
MBS		48	42	90	
	Pancreatic carcinoma	19	13	32	0.565[1]
	Cholangiocarcinoma	7	9	16	
	Ampullary carcinoma	8	5	13	
	Duodenal papilla carcinoma	7	7	14	
	Metastatic carcinoma	5	8	13	
	Gallbladder carcinoma	2	0	2	
BBS		19	24	43	
	Postoperative injury	11	12	23	0.914[1]
	Chronic pancreatitis	6	8	14	
	Others	2	4	6	

[1]Fisher's exact test. ARPS: Anti-reflux plastic stent; TPS: Traditional plastic stent; MBS: Malignant biliary stricture; BBS: Benign biliary stricture.

the ARPS group was significantly longer than that in the TPS group (210 d, SE 23.8, 95%CI: 163.4-256.6 *vs* 155 d, SE 6.0, 95%CI: 143.3-166.8; $P = 0.049$) (Table 3, Figure 5).

Other adverse events

Post-ERCP pancreatitis occurred in 4 (6.0%) patients and 2 (3.0%) patients in the ARPS and TPS groups, respectively ($P = 1.000$), which were mild and improved after conservative treatment. Post-ERCP cholangitis occurred in 1 (1.5%) patient in the ARPS group and 1 (1.5%) patient in the TPS group ($P = 1.000$). They were mild and relieved after antibiotic therapy. No stent proximal migration was observed. Stent distal migration was noted in 3 (4.5%) patients in the ARPS group, including 2 patients of partial migration and 1 patient of complete migration. There were 4 (6.0%) patients of distal migration in the TPS group, including 2 patients of partial migration and 2 patients of complete migration. No significant difference was noted in the incidence of distal migration between the two groups ($P = 0.718$). All partial distal migrated stents were retrieved endoscopically, and complete distal migrated stents passed spontaneously. No stent migration-related intestinal injury was observed (Table 3).

DISCUSSION

Our study revealed that placement of new ARPS in patients with biliary strictures could reduce the onset of stent-related cholangitis and prolong stent patency. No serious procedures or stents related adverse events were observed. The use of new ARPS proved to be safe and effective.

Although placement of SEMS is recommended for patients with unresectable MBS [22], many patients in our institution prefer to PSs rather than SEMSs due to the costs, especially those with life expectancy less than 6 mo. Therefore, PSs were used in this study for patients with MBS. Considering the limited patency of PSs, we scheduled routine stent exchange every 3 to 6 mo. However, some patients only underwent stent removal/exchange when cholangitis or stent dysfunction occurred, due to the large number of patients and long waiting time for admission in our institution.

In the present study, the occurrence of stent-related cholangitis in the ARPS group was significantly lower than that in the TPS group. Moreover, the median time and cumulative median time till the onset of first stent-related cholangitis were significantly later than those in the TPS group, which may be attributed to the anti-reflux mechanism. The anti-reflux valve of the new ARPS, designed as "duckbilled", could simulate the opening and closing function of a duck's bill. This design allows antegrade bile flow into the duodenum, while it closes immediately when the intestinal pressure increases, thereby reducing the reflux of intestinal contents such as bacteria and food materials. However, the role of new ARPS in reducing the onset of cholangitis was not obvious in the subgroup analyses. The sample size in this study

Table 3 Outcomes in the anti-reflux plastic stent group and traditional plastic stent group

	ARPS group, *n* = 67	TPS group, *n* = 66	*P* value
ERCP-related complications, *n* (%)	5 (7.5)	3 (4.5)	0.718[4]
Pancreatitis	4 (6.0)	2 (3.0)	1.000[4]
MBS/BBS	3/1	1/1	
Cholangitis	1 (1.5)	1 (1.5)	1.000[4]
MBS/BBS	1/0	1/0	
Stent-related cholangitis, *n* (%)	8 (11.9)	18 (27.3)	0.030[3]
MBS	7 (14.6)	13 (31.0)	0.078[3]
BBS	1 (5.3)	5 (20.8)	0.205[3]
Stent-related cholangitis, frequencies			0.115[4]
0	59	48	
1	6	12	
2	2	5	
3	0	1	
Time till the onset of first stent-related cholangitis, d[1]	128.5 (197.5); [65.0-347.0]	76.0 (72.8); [5.0-337.0]	0.039[5]
Stent distal migration, *n* (%)	3 (4.5)	4 (6.0)	0.718[4]
MBS/BBS	2/1	2/2	
Stent dysfunction, *n* (%)	44 (65.7)	52 (78.8)	0.091[3]
MBS/BBS	29/15	33/19	
Stent patency, d[2]	185.0 (8.6); [168.2-201.8]	133.0 (16.9); [99.8-166.2]	0.001[6]
MBS	173.0 (32.0); [110.3-235.7]	98.0 (11.6); [75.3-120.7]	0.008[6]
BBS	210.0 (23.8); [163.4-256.6]	155.0 (6.0); [143.3-166.8]	0.049[6]

[1]Median (interquartile range) [range].
[2]Median (standard error) [95% confidence interval].
[3]Chi-squared test.
[4]Fisher's exact test.
[5]Mann-Whitney *U* test.
[6]Log-rank test. ARPS: Anti-reflux plastic stent; TPS: Traditional plastic stent; ERCP: Endoscopic retrograde cholangiopancreatography; MBS: Malignant biliary stricture; BBS: Benign biliary stricture.

may not be adequate for exploratory subgroup analyses. A small number of patients in both groups experienced 2 to 3 episodes of stent-related cholangitis. These patients usually presented with mild cholangitis symptoms, such as fever (temperature > 38 °C) and chills, accompanied by upper abdominal discomfort, and without obvious signs of stent occlusion, namely non-stent dysfunction cholangitis. In the early stage of stent occlusion, bacteria from the duodenum refluxed into the biliary tract and colonized, thus causing mild cholangitis. Although antibiotics could ameliorate symptoms, mild cholangitis were prone to recur owing to the existence of partial stent occlusion. Therefore, patients with recurrent cholangitis after stent placement should be considered for exchange a new one.

As the relatively short stent patency has always been an important issue in clinical practice, prolonging stent patency was another focus of this and previous studies. The actual mechanisms of PS occlusion remain unclear. The initial event of PS occlusion is the biofilm formation[1,23]. Several previous studies[24-26] have focused on the material of stents or the special coat that prevent bacterial adherence and biofilm formation, revealing somewhat discrepant results between *in vitro* testing and clinical studies. Food materials refluxed from the duodenum have been observed in occluded PSs, thus suggesting that duodenobiliary reflux may have an important role in stent occlusion[1,8]. It could theoretically prolong stent patency if we change the design of stent to eliminate retrograde flow from the intestine. Our study confirmed that new ARPS was superior to TPS in achieving relatively longer stent patency in either BBSs

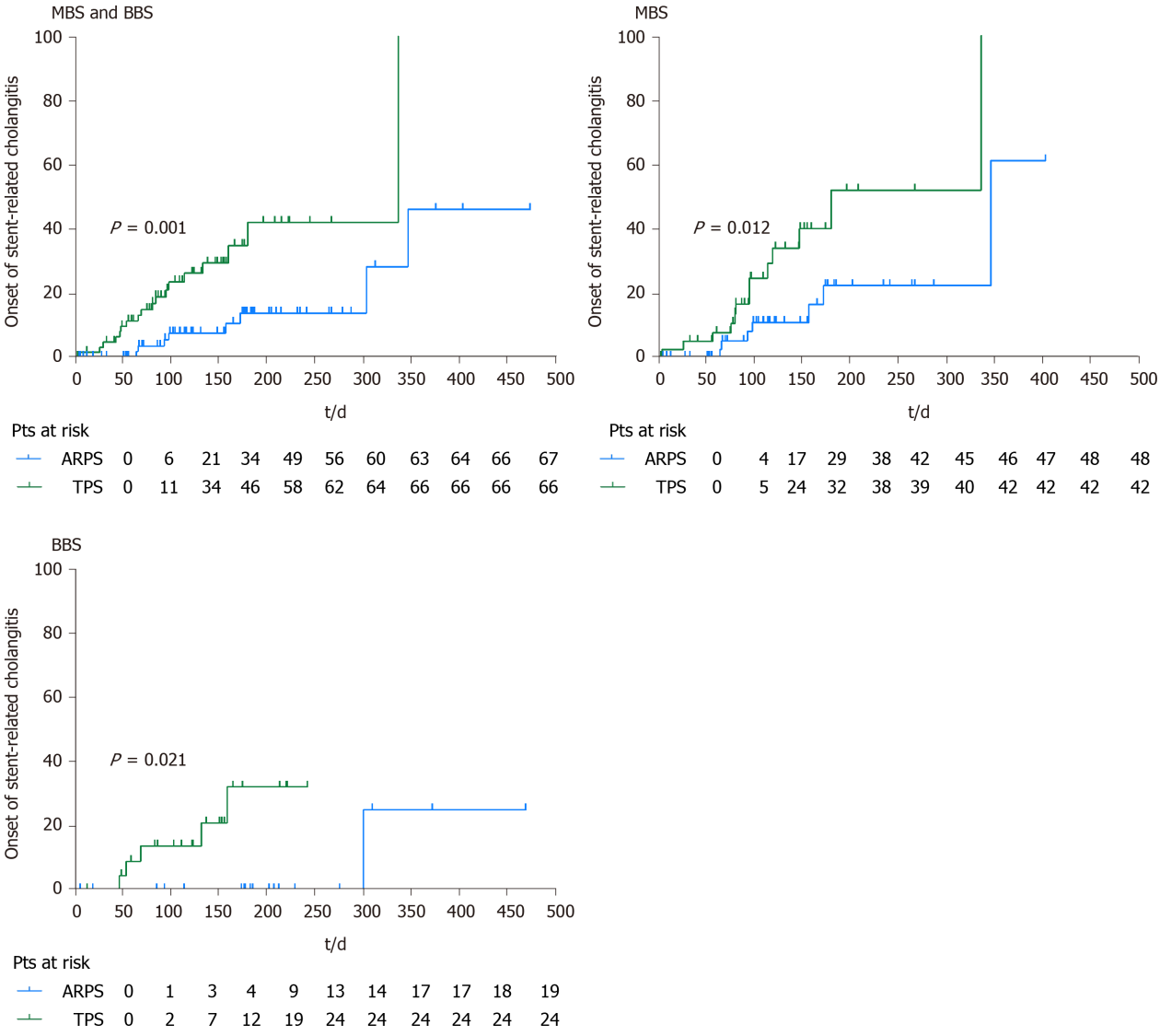


Figure 4 Kaplan-Meier curves of onset of stent-related cholangitis of anti-reflux plastic stent and traditional plastic stent (Log-rank test).
MBS: Malignant biliary stricture; BBS: Benign biliary stricture; Pts: Patients; ARPS: Anti-reflux plastic stent; TPS: Traditional plastic stent.

or MBSs. The ARPSs reported in prior studies[7,13,14] had a relatively large anti-reflux valve opening, which may diminish the effect on reducing duodenobiliary reflux. Moreover, the length of the anti-reflux valve was long (about 4cm), and the valve was easy to collapse or fold, resulting in poor bile drainage. Therefore, no ideal results were observed in their clinical trials. In the present study, the anti-reflux valve of the ARPS had a small opening, and its short length (about 1.5 cm) made it relatively hard to collapse or fold. It should be noted that the bile drainage gets blocked in the short term if the anti-reflux valve is not fully deployed during placement. This phenomenon occurred in 1 patient in our study; thus, complete expansion of the valve and sufficient bile flow are needed to be confirmed before the withdrawal of the duodenoscope.

Similar delivery systems were used in this study to deploy the investigated ARPS and TPS. Despite the valve, endoscopic placement of the ARPS was not difficult and did not increase the risk of biliary tract infection. The rates of post-ERCP cholangitis in the two groups were comparable to the rate (0.5-5%) previously reported[27-30]. European Society of Gastrointestinal Endoscopy guidelines[31] has shown that endoscopic sphincterotomy is a risk factor for post-ERCP pancreatitis; thus, endoscopic sphincterotomy was not routinely performed in this study. There was no significant difference in the incidence of post-ERCP pancreatitis between the two groups, neither this incidence was higher than the one (9.7%) reported in the latest meta-analysis[32]. Distal or proximal migration of PSs has been reported in 5%-10% of cases[21]. There was no proximal migration in our study. No significant difference was noted in the incidence of distal migration between the two groups, and our results were comparable to previous results (3%-6%)[21]. Previous studies[21,33] indicated

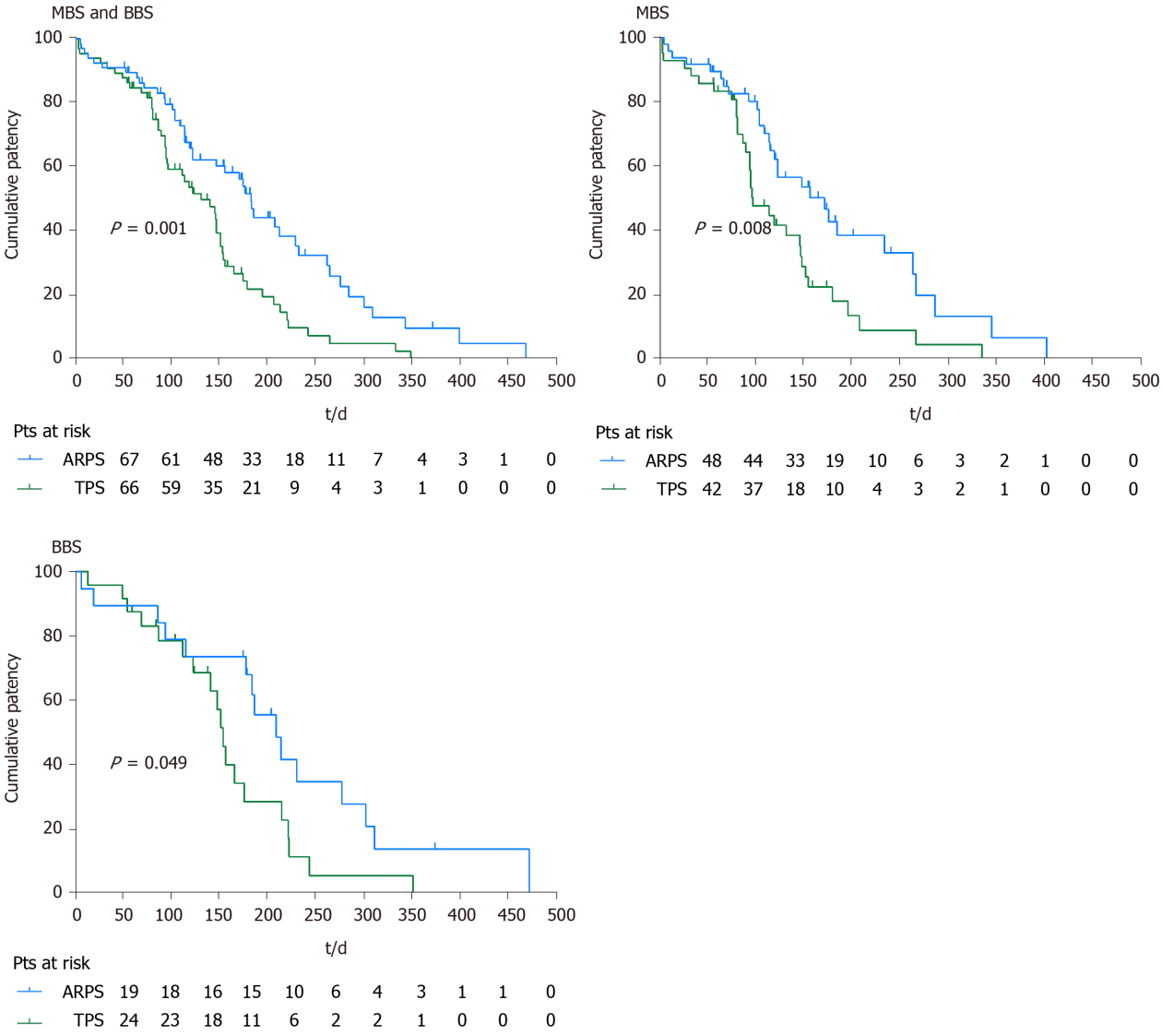


Figure 5 Kaplan-Meier curves of cumulative patency of anti-reflux plastic stent and traditional plastic stent (Log-rank test). MBS: Malignant biliary stricture; Pts: Patients; ARPS: Anti-reflux plastic stent; TPS: Traditional plastic stent.

that the most distal migrated stents can be retrieved endoscopically, and less than 1% of cases developed gut perforation caused by stent migration. No gut perforation was observed in our study. Therefore, the use of new ARPS in the treatment of biliary strictures does not increase the difficulty of procedures and the risk of adverse events related to the procedures or stents.

Some limitations of our study have to be mentioned. The first limitation was its retrospective design, but the data collected from a prospectively collected database supported the validity of the present results. The second limitation was that the ARPS and TPS used in this study exhibit different materials and antimigration designs. Previous studies have indicated that there were no significant differences in the rates of cholangitis and stent patency between stents made of different materials or stents with different antimigration designs[34-36]. Therefore, in the present study, the differences in the rates of stent-related cholangitis or stent patency between the two groups may not be attributed to those differences. However, further studies are needed to assess this hypothesis by comparing the same PS with or without anti-reflux valve. The third limitation was that macroscopic and microscopic examinations of dysfunctional stents were not performed, and the exact causes of stent dysfunction were unclear. The fourth limitation was the inadequate sample size for exploratory subgroup analyses. Further studies with large samples are needed to evaluate its safety and efficacy. The fifth limitation refer to potential selection bias in the inclusion of patients. Although patients with proximal biliary strictures or with pre-ERCP cholangitis were considered to have a higher risk of cholangitis, the distribution of these patients were comparable between the two groups in terms of baselines or

clinical outcomes.

CONCLUSION

In conclusion, our study suggests that it is safe and effective to use the new ARPS for the treatment of biliary strictures. This ARPS could reduce the risk of stent-related cholangitis and prolong stent patency. However, its role in reducing the onset of stent-related cholangitis is not obvious in the subgroup analyses. Studies with large samples are needed to evaluate further its safety and efficacy.

ARTICLE HIGHLIGHTS

Research background

Endoscopic biliary stenting is an established procedure for patients with biliary strictures. However, complications such as stent occlusion and stent-related cholangitis after stent placement may worsen the quality of patients' life. Since duodenobiliary reflux is a major cause of those complications, the design of stents with an anti-reflux valve is a potential solution for reducing duodenobiliary reflux.

Research motivation

The authors have previously developed a new anti-reflux plastic stents (ARPSs) and conducted a clinical trial to compare the patency of ARPSs with that of traditional plastic stents (TPSs), which showed this new ARPS had a significantly longer stent patency. However, the role of this new ARPS in reducing the onset of stent-related cholangitis compared with TPS remains unknown.

Research objectives

In this study, the authors compared this new ARPS with TPS in patients with biliary strictures.

Research methods

The authors retrospectively reviewed the data of consecutive patients with biliary strictures who underwent first endoscopic biliary stenting between February 2016 and May 2019 at West China Hospital, Sichuan University from our prospectively collected database. According to the inclusion and exclusion criteria, eligible patients were included for analysis. The onset of stent-related cholangitis, stent patency, clinical success, and other adverse events were evaluated.

Research results

During the study period, 67 patients in the ARPS group and 66 patients in the TPS group were included. There was a significant difference when comparing the onset of stent-related cholangitis, which was significantly lower in the ARPS group than in the TPS group. The cumulative median stent patency in the ARPS group was significantly longer than that in the TPS group. No significant differences were noted in the rates of clinical success and other adverse events between both groups.

Research conclusions

This new ARPS results in superior to TPS in reducing the risk of stent-related cholangitis and prolonging stent patency.

Research perspectives

Multicenter studies with large samples are expected to evaluate further the safety and efficacy of this new ARPS.

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ORIGINAL ARTICLE

Clinical Trials Study

Modified Xiaochaihu Decoction for gastroesophageal reflux disease: A randomized double-simulation controlled trial

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Abstract

BACKGROUND

Gastroesophageal reflux disease (GERD) has a high prevalence worldwide, and its incidence is increasing annually. Modified Xiaochaihu Decoction (MXD) could relieve the symptoms of GERD, but the effects of MXD on GERD manifestations and relapse prevention need to be further explained. Therefore, we performed a prospective, double-blind, and double-simulation study.

AIM

To verify the efficacy of MXD for GERD and its effect on esophageal motility.

METHODS

Using randomization, double-blinding, and a simulation design, 288 participants with GERD were randomized to the treatment group and control group and received herbs (MXD) plus omeprazole simulation and omeprazole plus herbs simulation, respectively, for 4 wk. The GERD-Q scale score and esophageal manometry were measured at baseline, after treatment, and at 1 mo and 3 mo follow-up visits when medication was complete to evaluate recurrence indicators.

RESULTS

The GERD-Q scale score in both groups decreased significantly compared to those before treatment ($P < 0.01$). However, no significant difference was observed

Clinical trial registration statement: This study is registered at: www.chictr.org.cn/; The registration identification number is ISRCTN17685397.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: All authors have no conflict of interest.

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between the two groups ($P > 0.05$). Esophageal manometry showed that participants with lower esophageal sphincter pressure reduction and the proportion of ineffective swallowing (more than 50%) improved in both groups from baseline ($P < 0.01$), especially in the treatment group ($P < 0.05$). The percentage of small intermittent contractions, large intermittent contractions, and increased pre-phase contractions in the treatment group significantly improved compared with baseline ($P < 0.05$) but did not improve in the control group ($P > 0.05$). There was no significant difference between the groups after treatment ($P > 0.05$). The percentage of weak esophageal contractility (distal contractile integral < 450 mmHg·s·cm), improved in both groups ($P < 0.01$), but no significant difference was observed between the groups after treatment ($P > 0.05$). The relapse rate in the treatment group was lower than that in the control group at the 1 mo ($P < 0.01$) and 3 mo follow-up ($P < 0.05$).

CONCLUSION

MXD has a similar therapeutic effect to omeprazole in mild-to-moderate GERD. The therapeutic effect may be related to increased pressure in the lower esophageal sphincter and reduced ineffective swallowing.

Key Words: Gastroesophageal reflux disease; Traditional Chinese medicine; Esophageal sphincter pressure; Gastroesophageal reflux disease-Q scale score; Modified Xiaochaihu Decoction

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Core Tip: This multicenter, randomized, double-blind, double-simulation study proved that Modified Xiaochaihu Decoction has a similar therapeutic effect to omeprazole in the treatment of patients with typical symptoms of gastroesophageal reflux disease and reflux esophagitis grades A and B. Modified Xiaochaihu Decoction was superior to omeprazole in improving lower esophageal sphincter resting pressure and reducing ineffective esophagus swallowing. The recurrence rate of symptoms was significantly lower than that of omeprazole within 1 mo and 3 mo after completing treatment. Modified Xiaochaihu Decoction may be an alternative treatment to proton pump inhibitor maintenance in patients with gastroesophageal reflux disease.

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INTRODUCTION

Gastroesophageal reflux disease (GERD) has a high prevalence worldwide, and its incidence is increasing annually[1]. Impaired esophageal and gastric motor function as well as reduced resting pressure of the lower esophageal sphincter are considered to be involved in the pathogenesis of GERD. Chemical drugs can help relieve the symptoms and eliminate inflammation, but the relapse rate is high. It is reported that esophagitis and other symptoms are 80% and 90%, respectively, 6 mo after proton pump inhibitor (PPI) withdrawal[2]; therefore, many patients require long-term maintenance medication. Our previous observations showed that Modified Xiaochaihu Decoction (MXD) could relieve the symptoms of GERD[3]. The present study aimed to evaluate the effects of MXD on GERD manifestations and relapse prevention and to determine its underlying mechanism, from the aspect of esophageal motion, using a prospective, double-blind, and double-simulation design compared with omeprazole.

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MATERIALS AND METHODS

Patients

This is a prospective, double-blind, and double-simulation study, which was sponsored by Beijing Hospital of Traditional Chinese Medicine of Capital Medical University and conducted in Peking Union Medical College Hospital, Beijing Shijitan Hospital of Capital Medical University, and Shengjing Hospital of China Medical University from January 2015 to December 2019.

Ethical permission

The study was reviewed and approved by the Ethics Committee of Beijing Hospital of Traditional Chinese Medicine of Capital Medical University. The trial registration number is ISRCTN17685397.

Diagnostic criteria

Participants who met the standard of the "Chinese Experts Opinion on Gastroesophageal Reflux Disease"[2] were considered for inclusion in the study.

The inclusion criteria were: (1) patients aged 18-65 years; (2) satisfied the diagnostic criteria for GERD; and (3) GERD-Q score ≥ 8, with A-B esophagitis under gastroscopy.

Exclusion criteria were: (1) a history of gastric, esophageal, and duodenal surgery; (2) presence of Zollinger-Ellison syndrome or primary esophageal motility disease; (3) suspected or confirmed to have a malignant disease or have early warning symptoms; (4) use of PPI or H2 receptor blockers within 2 wk before enrollment; (5) serious primary heart, liver, lung, kidney, pancreas, or other serious diseases that would affect their survival; (6) disabled patients (blind, deaf, dumb, mentally retarded, mentally disabled, physically disabled) as required by law; (7) suspected or confirmed history of alcohol and drug abuse; (8) allergies, such as those with a history of allergies to two or more drugs or food; or known allergies to the ingredients of MXD; and (9) pregnant and lactating women.

Sample size estimation

The sample-size calculation in this design was based on a non-inferiority test with a 1:1 comparison principle. Using the one-sided test, according to previous literature reports and research results, the formula: [n1 = n2 = 2 (Z_α + Z_β) 2P (1-P)/δ2, Z_α = 1.645, Z_β = 0.845, P = 0.65, δ = -0.15; n1 = n2 approximately 125] was used. In this study, the expulsion rate was designed to be 15%, the sample content required for each group was 144, and a total of 288 samples were required.

Randomization and allocation concealment

Randomization into the treatment or control group was performed with a 1:1 allocation ratio. Balanced treatment assignments were achieved by block randomization. This process was performed using SAS 9.4 software to generate a random sequence. The block length was eight. Now that there were four units and a total of 288 subjects, each unit was assigned 72 connecting consecutive codes and the corresponding allocation to treatment or control.

Participants in the treatment group were given herbal granules (2 packets/d, one packet at a time with 200 mL water, before meals) combined with omeprazole simulation tablets produced by Jiangyin Tianjiang Pharmaceutical Co., Ltd. The traditional Chinese medicine (TCM) prescriptions were mainly composed of *Bupleurum* 10 g, *Codonopsis* 15 g, fried *Atractylodes* 12 g, *Coptis chinensis* 10 g, *Flos* insulae 10 g, and fried Raphani 15 g. Omeprazole placebo was produced by Lunan New Times Pharmaceutical Co., Ltd.) and was taken orally, 20 mg each time, once on an empty stomach in the morning.

Participants in the control group were given omeprazole enteric-coated tablets combined with an herbal granule placebo. The herbal granule placebo was produced by Jiangyin Tianjiang Pharmaceutical Co., Ltd. The simulated MXD placebo contained 2.5% of the dose of the original formula, with a color correction agent, seasoning agent, starch, dextrin, and other auxiliary materials added. After spray drying, crushing, screening, mixing, granulation, and packaging, the granule simulation agent was placed in bags. The appearance, characteristics, and odor were the same as those of the actual herbal medicine and contained 10.3 g/bag (produced by Jiangyin Tianjiang Pharmaceutical Co., Ltd.). Omeprazole enteric-coated tablets (Lunan New Times Pharmaceutical Co., Ltd., SFDA approval No. 008140505) were taken orally, 20 mg each time, once on an empty stomach in the morning.

All medications in both groups were administered for 4 wk, and all participants had a washout period of 2 wk before taking the medication.

Blinding

The blind codes were sealed separately and kept by those who were not directly involved in this clinical trial. Doctors and patients were blind to the medicine. The medicine and placebos were packaged in the same outer packaging, and their appearance, color, and characteristics were consistent. Both were made by the same manufacturer.

Esophageal manometry

The ManoScan360TM gastrointestinal dynamic high-resolution esophageal manometry system (Given Imaging, United States) and a solid-state surrounding pressure measuring electrode catheter with 36 pressure measuring channels was used. Adjacent channels were spaced 1 cm apart, and each channel had 12 surround pressure points. The recorded data were analyzed with ManoView Analysis software.

The patient underwent a high-resolution manometry test after fasting for at least 8 h, and the pressure-measuring catheter was inserted through the nasal cavity. The depth of the catheter was adjusted so that the display screen showed two high-pressure areas at the proximal and distal ends of the esophagus, the upper esophageal sphincter, and the lower esophageal sphincter (LES), and the catheter was fixed at the nasal wing. During the examination, the patient was placed in the supine position and adapted for 5 min. The basal pressure level in the esophagus was recorded, and the patient swallowed 10 times with 5 mL each time. The interval between two swallows was at least 30 s, and the patient remained awake throughout the recording.

Study endpoints

Primary endpoints: The primary endpoint of this study was the GERD-Q scale score. Positive symptom scoring may reflect the frequency of heartburn and reflux symptoms and scored 0, 1, 2, 3 points according to "0 d," "1 d," "2-3 d," or "4-7 d" with a maximum score of 6 points. Negative symptom scoring may reflect the frequency of central abdominal pain and nausea and scored 3, 2, 1, 0 points according to "0 d," "1 d," "2-3 d," or "4-7 d" with a maximum score of 6. Positive impact scoring may reflect the frequency of heartburn or reflux affecting sleep and the frequency of patients taking extra over-the-counter drugs such as antacids and scored 0, 1, 2, 3 points according to "0 d," "1 d," "2-3 d," and "4-7 d" with a maximum score of 6 points. The highest total score of the 6 questions was 18 points. Scores were recorded before medication and at the second and fourth weeks of medication, and follow-up scores were recorded at the first and third month after medication was complete.

Secondary endpoints: Lower esophageal sphincter pressure (LESP), the percentage of ineffective swallowing > 50%, percentage of small peristaltic interruption, percentage of large peristaltic interruption, distal contractile integral (DCI), and percentage of early contractions were the secondary endpoints. As not all participants in the study had abnormal esophageal manometry indicators, only those with abnormalities at baseline were measured before and after completion of medication. Indices of esophageal manometry were recorded before and 4 wk after treatment.

Follow-up

Follow-up was performed at 1 mo and 3 mo after completion of medication, and the number and percentage of patients who were not on medication, on maintenance medication, intermittent medication, and on-demand medication were recorded in the treatment group and the control group.

Statistical analysis

SAS9.4 software (Beijing Hospital of TCM Version, Order Number: 9C1XJD) was used for statistical analysis. mean ± SD were used to describe the continuous variables. Frequency and percentage were used to describe the categorical variables. Statistical tests were performed using the bilateral test, and a P value < 0.05 was considered statistically significant. Measurement data were analyzed using the t-test or Wilcoxon rank-sum test. For comparisons within groups, the paired t-test or Wilcoxon signed-rank test was used. For comparisons between groups, categorical variables were analyzed using the χ^2 test and Fisher's exact test, and continuous variables were analyzed using analysis of variance if normally distributed, or using the nonparametric test if non-normally distributed. For repeated measurements at several time

points between groups, the repeated measures analysis of variance or the generalized estimating equation was used.

RESULTS

Patient characteristics

A total of 288 patients were included in this study and were divided into the treatment group and the control group, with 144 cases in each group. Twenty-eight patients in the treatment group were excluded, including 1 patient with adverse reactions (systemic pruritus after taking the medicine, and the symptoms subsided after 2 d of withdrawal), 14 cases left the study due to personal reasons, and 13 cases left due to dissatisfaction with the treatment; of the 14 patients excluded in the control group, 9 patients left the study due to personal reasons, 5 patients left due to dissatisfaction, and a total of 246 patients completed the study (Figure 1).

One hundred and sixteen patients were included in the treatment group, 50 males and 66 females, aged 18-65 years, with an average age of 50.30 ± 10.38 years. Disease course ranged from 1 mo to 36.67 years. One hundred and thirty patients were included in the control group, 64 males and 66 females, aged 24-65 years, with an average age of 50.48 ± 11.71 years. Disease course ranged from 2 mo to 20 years. There were no statistically significant differences between the two groups of patients in terms of gender, age, and duration of illness.

GERD-Q scale scoring

There were significant differences ($P < 0.01$) within the treatment group in relation to withdrawal of medication before treatment and treatment at 2 wk, 4 wk, 1 mo, and 3 mo. In the control group there were significant differences ($P < 0.01$) in relation to withdrawal of medication before treatment compared with treatment at 2 wk, 4 wk, 1 mo, and 3 mo. There were no significant differences between the treatment group and the control group during treatment and follow-up visits (Table 1).

Indicators of esophageal manometry

With regard to post-treatment changes in patients with reduced LESP before treatment, post-treatment LESP was higher than pre-treatment LESP in both the treatment group and control group, and there was a significant difference between the groups ($P < 0.01$); there was also a significant difference between the two groups after treatment ($P < 0.01$) (Table 2 and Figure 2).

Changes in patients with small intermittent contractions

The post-treatment proportion of small intermittent contractions in the treatment group was reduced compared with that before treatment, and the difference was statistically significant ($P < 0.05$). There was no significant difference within the control group before and after treatment ($P > 0.05$), and there was no significant difference between the two groups after treatment ($P > 0.05$) (Table 3).

Changes in patients with increased large intermittent contractions

The post-treatment proportion of large intermittent contractions in the treatment group was reduced compared with that before treatment, and the difference was statistically significant ($P < 0.05$). There was no significant difference within the control group before and after treatment ($P > 0.05$), and there was no significant difference between the two groups after treatment ($P > 0.05$) (Table 4).

Changes in patients with ineffective swallowing > 50%

The post-treatment proportion of ineffective swallowing in the treatment group and control group was reduced compared with that before treatment. The difference was statistically significant in the two group ($P < 0.01$), and the difference was statistically significant in the two groups after treatment ($P < 0.05$) (Table 5).

Changes in patients with pre-phase contractions

The post-treatment proportion of pre-phase contractions in the treatment group was reduced compared with that before treatment, and the difference was statistically significant ($P < 0.05$). There was no significant difference within the control group before and after treatment ($P > 0.05$), and there was no significant difference between the two groups after treatment ($P > 0.05$) (Table 6).

Table 1 Gastroesophageal reflux disease-Q scale scoring changes

	Treatment, *n* = 116	Control, *n* = 130	Mean difference (95% confidence interval)	*P* value
Before treatment	10.53 ± 2.01	10.10 ± 1.64	0.434 (0.067 to 0.077)	0.077
2 wk treatment	8.25 ± 1.85[a]	7.99 ± 1.85[a]	0.266 (0.100 to 0.112)	0.108
4 wk treatment	7.36 ± 1.71[a]	7.21 ± 1.63[a]	0.147 (0.463 to 0.483)	0.469
1 mo withdrawal	7.59 ± 1.82[a]	7.59 ± 1.47[a]	0.003 (0.301 to 0.309)	0.301
3 mo withdrawal	7.56 ± 1.78[a]	7.44 ± 1.54[a]	0.114 (0.826 to 0.841)	0.837

[a]*P* < 0.01, 2 wk treatment, 4 wk treatment, 1 mo of withdrawal, 3 mo of withdrawal were compared with before treatment.

Table 2 Changes in patients with reduced lower esophageal sphincter pressure

	Treatment, *n* = 39	Control, *n* = 41	*P* value
Pre-treatment, mmHg	7.99 ± 2.92	8.26 ± 2.97	0.646
Post-treatment, mmHg	16.15 ± 4.90[a,b]	13.23 ± 3.71[a]	0.003
Mean difference (95% confidence interval)	-8.158 (0.000 to 0.000)	-4.917 (0.000 to 0.000)	
P value	0	0.000[a]	

[a]*P* < 0.01, compared with pre-treatment in the group.
[b]*P* < 0.05, compared with the control group after treatment.

Table 3 Changes in patients with small intermittent contractions

	Treatment, *n* = 52	Control, *n* = 49	*P* value
Pre-treatment, %	30.48 ± 10.39	33.67 ± 9.50	0.077
Post-treatment, %	27.11 ± 8.47[b]	29.79 ± 8.77	0.063
Mean difference (95% confidence interval)	3.365 (0.035 to 0.043)	3.469 (0.057 to 0.066)	
P value	0.037	0.051	

[b]*P* < 0.05, compared with pre-treatment in the group.

Table 4 Changes in patients with large intermittent contractions

	Treatment, *n* = 7	Control, *n* = 9	*P* value
Pre-treatment, %	22.85 ± 7.55	18.88 ± 6.00	0.261
Post-treatment, %	17.14 ± 4.87[b]	15.55 ± 5.27	0.529
Mean difference (95% confidence interval)	5.714 (0.119 to 0.132)	3.333 (0.237 to 0.254)	
P value	0.046	0.083	

[b]*P* < 0.05, compared with pre-treatment in the group.

Changes in patients with DCI less than 450 mmHg·s·cm

The post-treatment value of DCI in the treatment group and control group both increased compared with the values before treatment, and the difference was statistically significant in the group ($P < 0.01$). There was no significant difference in the two groups after treatment ($P > 0.05$) (Table 7).

Table 5 Changes in patients with ineffective swallowing > 50%

	Treatment, *n* = 49	Control, *n* = 48	*P* value
Pre-treatment, %	85.30 ± 15.42	83.12 ± 17.03	0.647
Post-treatment, %	63.46 ± 21.07[a,b]	72.29 ± 21.52[a]	0.016
Mean difference (95% confidence interval)	21.837 (0.000 to 0.000)	10.833 (0.002 to 0.004)	
P value	0	0.003	

[a]*P* < 0.01, compared with pre-treatment in the group.
[b]*P* < 0.05, compared with the control group after treatment.

Table 6 Changes in patients with increased pre-phase contractions

	Treatment, *n* = 21	Control, *n* = 25	*P* value
Pre-treatment, %	24.52 ± 8.04	26.00 ± 8.16	0.503
Post-treatment, %	19.04 ± 6.24[b]	23.20 ± 7.48	0.052
Mean difference (95% confidence interval)	5.476 (0.025 to 0.032)	2.800 (-1.894 to 7.494)	
P value	0.035	0.23	

[b]*P* < 0.05, compared with pre-treatment in the group.

Table 7 Changes in patients with distal contractile integral less than 450 mmHg·s·cm

	Treatment, *n* = 23	Control, *n* = 23	*P* value
Pre-treatment, mmHg·s·cm	237.80 ± 87.62	243.97 ± 86.53	0.373
Post-treatment, mmHg·s·cm	417.53 ± 128.22[b]	400.26 ± 136.63[b]	0.111
Mean difference (95% confidence interval)	-179.731 (0.000 to 0.000)	-156.283 (0.000 to 0.000)	
P value	0	0	

[b]*P* < 0.05, compared with pre-treatment in the group.

Efficacy in reducing recurrence rate

Patients were followed up at 1 mo after completing treatment, and a significant difference was observed between the treatment group and the control group ($P < 0.01$) (Table 8).

Patients were followed up at 3 mo after completing treatment, and a significant difference was observed between the treatment group and the control group ($P < 0.05$) (Table 9).

DISCUSSION

This multicenter, randomized, double-blind, double-simulation study proved that MXD has a similar therapeutic effect to omeprazole in the treatment of patients with typical symptoms of GERD and reflux esophagitis grades A and B. MXD was superior to omeprazole in improving LES resting pressure and reducing ineffective esophagus swallowing. The recurrence rate of symptoms was significantly lower than that of omeprazole within 1 mo and 3 mo after completing treatment. MXD may be an alternative treatment to PPI maintenance in patients with GERD.

GERD-Q scale scoring has high diagnostic accuracy in GERD and can evaluate quality of life and treatment response[4-6]. The results of this study showed that the GERD-Q scale scores in the two groups revealed significant changes before and after treatment, while there was no significant difference between the two groups indicating that MXD and the PPI omeprazole had equivalent clinical efficacy in improving the

Table 8 Follow-up results at 1 mo after completing treatment, *n* (%)

	Treatment, *n* = 116	Control, *n* = 130	*P* value
Not on medication	97 (83.6)[b]	86 (66.2)	0.002
Maintenance medication	12 (10.3)	16 (12.3)	-
Intermittent medication	1 (0.9)	14 (10.8)	-
On-demand medication	6 (5.2)	14 (10.8)	-

[b]$P < 0.05$, compared with pre-treatment in the group.

Table 9 Follow-up results at 3 mo after completing treatment, *n* (%)

	Treatment, *n* = 116	Control, *n* = 130	*P* value
Not on medication	85 (73.3)[b]	71 (54.6)	0
Maintenance medication	14 (12.1)	25 (19.2)	
Intermittent medication	6 (5.2)	9 (6.9)	
On-demand medication	11 (9.5)	25 (19.2)	

[b]$P < 0.05$, compared with pre-treatment in the group.

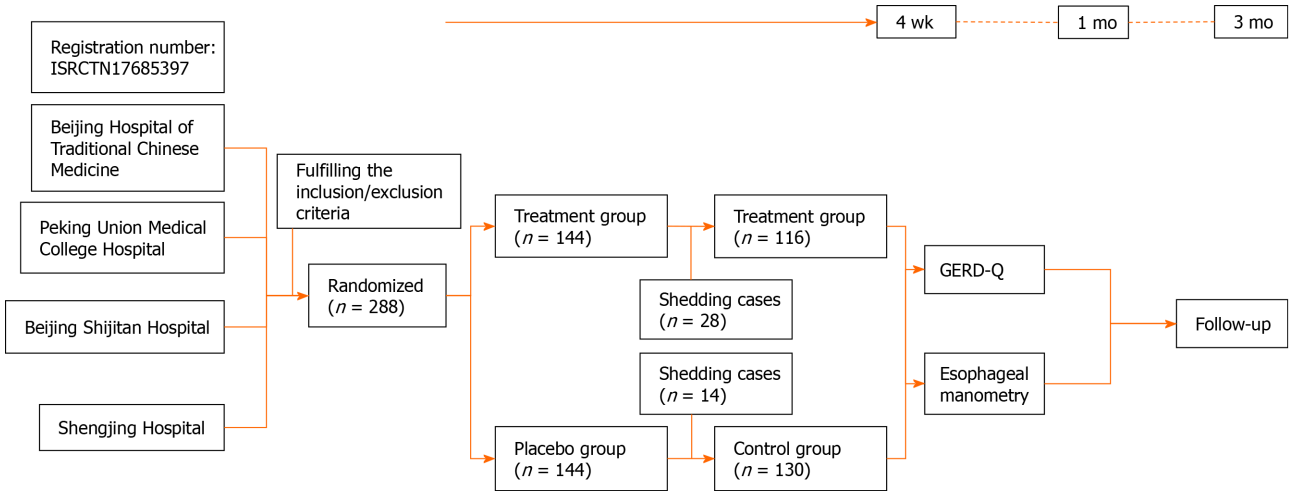


Figure 1 Study flow chart. GERD: Gastroesophageal reflux disease.

symptoms of GERD.

Esophageal dysfunction is a common cause of GERD. Abnormal dysfunction is mainly reflected in the weakened esophageal anti-reflux barrier function and esophageal clearance function. This study only compared abnormal esophageal motility indicators before and after treatment to explore the possible mechanism of MXD in the treatment of GERD. The results showed that MXD increased the LES resting pressure, enhanced the esophageal barrier effect, reduced ineffective contractions to improve esophageal clearance, reduced pre-phase contractions to adjust the coordination of esophageal movement, and improved esophageal clearance function. LES is an important component of the esophageal anti-reflux barrier, and its pressure is higher than that in the stomach to prevent the reflux of gastrointestinal content into the esophagus. It has been confirmed that LESP in GERD patients is significantly lower than in normal individuals[7]. When LESP decreases, the contents of the stomach and duodenum are more easily refluxed into the esophagus.

The research by Hu *et al*[8] showed that LES pressure decreases with the severity of acid reflux, and the decrease in LESP results in a significant increase in the incidence of hiatal hernia. Food in the esophagus is mainly propelled by peristalsis. When

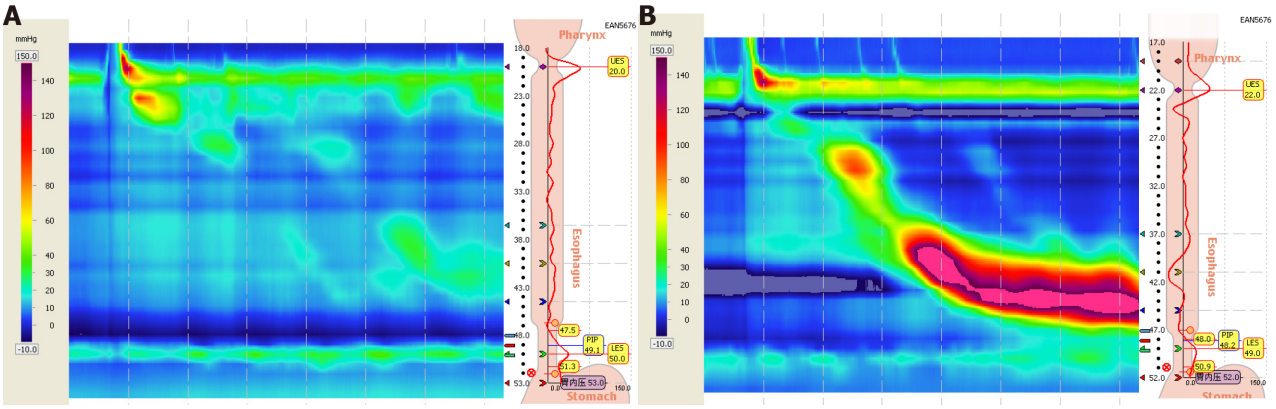


Figure 2 High-resolution esophageal manometry before and after treatment. The results in A and B were from the same patient in the treatment group. A: High-resolution esophageal manometry before treatment; B: High-resolution esophageal manometry 3 mo after treatment.

peristalsis is interrupted, the transmission and clearance functions of the esophagus in terms of the food bolus and reflux content are impaired, resulting in weakened clearance of the esophagus. Ribolsi *et al*[9] confirmed that esophageal peristalsis interruption in patients with GERD is associated with increased acid exposure in the supine position and increased reflux clearance time.

The Chicago esophageal dyskinesia classification released in 2015 defined swallowing with DCI < 450 mmHg·s·cm as ineffective swallowing and ineffective esophageal motility as > 50% of ineffective swallowing[10]. Frequent ineffective peristalsis of the esophagus can lead to impaired esophageal clearance and prolong the exposure duration of the food bolus and reflux content in the esophagus[11], thereby stimulating reflux symptoms. Liu *et al*[12] showed that GERD symptoms were closely related to DCI and ineffective esophageal peristalsis. With the widespread application of pH monitoring, the results of existing research show that ineffective esophageal peristalsis will lead to longer reflux time in both the supine and upright position and prolong average acid clearance time[8,13]. In addition, the pre-phase contractions of the esophagus will block passage of the food bolus and impair the peristaltic function of the esophagus. If the food bolus remains in the esophagus for an extended period, it will also increase stimulation of the esophageal mucosa. It can be inferred that MXD improved the patients' symptoms *via* the above effects.

The difficulty in the treatment of GERD lies in its high recurrence rate. Some studies have shown that in patients with GERD who take PPIs for 4-8 wk that the recurrence rate is 26.0%-47.8%, and the recurrence rate is 30.4% during the 1 year follow-up[14-16]. The follow-up visits in the present study were conducted at 1 mo and 3 mo after completing treatment. Patients not on medication, on maintenance medication, intermittent medication, and on-demand medication were assessed. It was found that the recurrence rate following TCM was low, and its ongoing efficacy was significantly better than omeprazole. The results of this study indicated that by increasing the resting pressure of the LES, improving ineffective swallowing, and reducing pre-phase contractions, the mechanism of reflux is partially corrected, and MXD can lower the recurrence rate.

It is generally accepted that the pathophysiology of GERD mainly involves inflammation and the immune response[17-19]. Modern pharmacological studies have shown that MXD has anti-inflammatory, antioxidant, and immunomodulatory activity. Saikoside, the active component of bupleurum has significant anti-inflammatory, immunoregulatory, and neuroregulatory effects[20]. Studies have shown that saikoside can significantly reduce the expression of tumor necrosis factor-α, interleukin-1, and interleukin-6 and can inhibit the production of nitric oxide, thereby contributing to its anti-inflammatory effects, and saikoside is an effective NF-κB channel inhibitor[21,22]. Studies have shown that inulin fructan extracted from the roots of Codonopsis has anti-inflammatory and anti-oxidant effects. Li *et al*[23] studied the protective effect of inulin fructan on ethanol-induced acute gastric ulcers in rats, and the results showed that inulin fructan significantly increased the activity of superoxide dismutase and glutathione peroxidase in gastric tissue in a dose-dependent manner and reduced the content of malondialdehyde and nitric oxide, thereby protecting the gastric mucosa. In addition, the active component Berberine from *Coptis chinensis* also has similar anti-inflammatory and antioxidant effects[24], and it has been shown that Berberine has a killing effect on *Helicobacter pylori*[25].

CONCLUSION

This study had the following limitations: the research only compared clinical symptoms, and pH monitoring before and after treatment was not carried out. In addition, the treatment duration was 4 wk, which was insufficient. It is considered that the course of treatment should be extended to 8 wk as this would be more conducive to symptom relief and resolution of inflammation. The next phase in this research is to extend the course of treatment and carry out in-depth observations on the effect of MXD on acid reflux.

In the present study, the number of drop-outs in the TCM group was higher, most of which were due to severe acid-refractory heartburn symptoms, and the TCM did not take effect quickly. Due to the double-blind and double-simulation design of the study, we believe that the results objectively demonstrate the effects of MXD and omeprazole on clinical symptoms and esophageal dynamics in GERD patients and provide evidence-based data for GERD patients to receive further treatment. Therefore, it is recommended that patients with reflux symptoms without serious or reflux esophagitis belonging to class A-B be treated with TCM, and for patients with severe symptoms of acid reflux and heartburn, which require quick relief, both Chinese and Western medicine is suggested to obtain more satisfactory efficacy.

ARTICLE HIGHLIGHTS

Research background

Gastroesophageal reflux disease (GERD) has a high prevalence worldwide, and its incidence is increasing annually. According to the preliminary experiment of the research group, Modified Xiaochaihu Decoction (MXD) could relieve the symptoms of GERD.

Research motivation

The effects of MXD on GERD manifestations and relapse prevention need to be further explained. Therefore, we performed a prospective, double-blind, and double-simulation study.

Research objectives

To verify the efficacy of MXD for GERD and its effect on esophageal motility.

Research methods

Using randomization, double-blinding, and a simulation design to compare the GERD-Q scale score and esophageal manometry between patients under the treatment of MXD (treatment group) and omeprazole (control group).

Research results

In total, 288 patients were divided into the treatment group and control group. The GERD-Q scale score in both groups decreased significantly compared to those before treatment ($P < 0.01$). However, no significant difference was observed between the two groups ($P > 0.05$). Esophageal manometry showed that participants with sphincter pressure reduction and the proportion of ineffective swallowing (more than 50%) improved in both groups from baseline ($P < 0.01$), especially in the treatment group ($P < 0.05$). The percentage of small intermittent contractions, large intermittent contractions, and increased pre-phase contractions in the treatment group significantly improved compared with baseline ($P < 0.05$) but did not improve in the control group ($P > 0.05$). The percentage of weak esophageal contractility (distal contractile integral < 450 mmHg·s·cm) improved in both groups ($P < 0.01$). The relapse rate in the treatment group was lower than that in the control group at the 1 mo ($P < 0.01$) and 3 mo follow-up ($P < 0.05$).

Research conclusions

MXD has a similar therapeutic effect to omeprazole in mild-to-moderate GERD. The therapeutic effect may be related to increased pressure in the lower esophageal sphincter and reduced ineffective swallowing.

Research perspectives

Our results supported that MXD has a similar therapeutic effect to omeprazole in mild-to-moderate GERD and could improve esophageal motility.

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ORIGINAL ARTICLE

Observational Study

Relationship between clinical features and intestinal microbiota in Chinese patients with ulcerative colitis

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Abstract

BACKGROUND

Dysbacteriosis may be a crucial environmental factor for ulcerative colitis (UC). Further study is required on microbiota alterations in the gastrointestinal tract of patients with UC for better clinical management and treatment.

AIM

To analyze the relationship between different clinical features and the intestinal microbiota, including bacteria and fungi, in Chinese patients with UC.

METHODS

Eligible inpatients were enrolled from January 1, 2018 to June 30, 2019, and stool and mucosa samples were collected. UC was diagnosed by endoscopy, pathology, Mayo Score, and Montreal classification. Gene amplicon sequencing of 16S rRNA gene and fungal internal transcribed spacer gene was used to detect the intestinal microbiota composition. Alpha diversity, principal component analysis, similarity analysis, and Metastats analysis were employed to evaluate differences among groups.

RESULTS

A total of 89 patients with UC and 33 non-inflammatory bowel disease (IBD) controls were enrolled. For bacterial analysis, 72 stool and 48 mucosa samples were obtained from patients with UC and 21 stool and 12 mucosa samples were obtained from the controls. For fungal analysis, stool samples were obtained from 43 patients with UC and 15 controls. A significant difference existed between the fecal and mucosal bacteria of patients with UC. The α-diversity of intestinal bacteria and the relative abundance of some families, such as *Lachnospiraceae* and *Ruminococcaceae*, decreased with the increasing severity of bowel inflammation, while *Escherichia-Shigella* showed the opposite trend. More intermicrobial correl-

ations in UC in remission than in active patients were observed. The bacteria-fungi correlations became single and uneven in patients with UC.

CONCLUSION

The intestinal bacteria flora of patients with UC differs significantly in terms of various sample types and disease activities. The intermicrobial correlations change in patients with UC compared with non-IBD controls.

Key Words: Ulcerative colitis; Intestinal microbiota; Intermicrobial correlation; Bacteria; Fungi; Chinese

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Core Tip: Further study is required on gut microbiota alterations in patients with ulcerative colitis (UC). This study aimed to analyze the relationship between clinical features and the intestinal microbiota, including bacteria and fungi, in Chinese patients with UC. Gene amplicon sequencing of 16S rRNA gene and fungal internal transcribed spacer gene was applied. A total of 89 UC patients and 33 controls were enrolled. Results showed that the intestinal bacteria flora of patients with UC differed significantly in terms of various sample types and disease activities. The intermicrobial correlations changed in patients with UC compared with controls.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a group of chronic inflammatory disorders of the gastrointestinal tract that are divided into ulcerative colitis (UC), Crohn's disease, and IBD-unclassified[1]. The worldwide incidence rate of IBD varies greatly[2]. Among countries in the Asia-Pacific area, where the rate of IBD has rapidly increased, China has the highest incidence, with UC being the most common type[3]. The etiology of UC is still unclear, and it may be related to an abnormal epithelial barrier function and immune response driven by environmental factors in individuals with genetic susceptibility[1].

Disordered intestinal microbiota, or dysbacteriosis, is considered one of the crucial environmental factors for IBD[1]. Animal experiments have indicated the association between microbiota and UC. Some animal models, including mice with an immune deficiency, spontaneously develop chronic colitis resembling human UC[4]. However, under germ-free conditions or durative antibiotic therapy, the development of spontaneous colitis in these mice was prevented[4]. Dozens of studies found that bacterial strains derived from mice with colitis or patients with IBD could induce intestinal inflammation in mice with an immune deficiency and even in wild-type mice[4].

Existing evidence from animal experiments has stressed the necessity of a thorough understanding of the constitution and changes of the intestinal microbiota in patients with UC to elucidate the possible pathogenesis and investigate potential treatments. Numerous studies have focused on the variations in bacterial diversity and composition. Compared with non-IBD controls, there is a trend of decreased microbial α-diversity in patients with UC[5-7], although the total bacterial count decreased or increased in different studies[8,9]. Altered bacterial composition in patients with UC was discovered in almost all the related studies. The intestinal bacterial microbiota in the feces was different from that in the colon mucosa[10,11]. Differences were also found between patients with UC and non-IBD controls[6,7,12-16], among patients with UC of various severity[9,17,18] or disease types[11,19], between inflamed and noninflamed biopsies[6,16,19,20], among patients who accepted different therapies[10,

study participants provided informed written consent prior to study enrollment.

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11], and between patients who responded or did not respond to certain therapies[13]. In recent years, studies have also focused on changes in intestinal fungi in IBD patients [5,17]. However, existing studies concerning the above areas have arrived at various conclusions, which may be attributed to the limited sample size, imprecise stratification of patients, or other factors. Therefore, further study is required to obtain more information about microbiota alterations in the gastrointestinal tract of patients with UC for better clinical management and treatment.

In this study, our main purpose was to characterize the intestinal microbiota of patients with UC by 16S rRNA gene amplicon sequencing and compare the differences between the fecal and mucosa samples of the same UC patient, among patients with active UC, those with UC in remission, and non-IBD controls, among patients with different degrees of disease activity or with different disease types, and between biopsy samples collected from inflamed and noninflamed sites. In addition, the fecal fungal microbiota in the patients and controls was also analyzed using internal transcribed spacer (ITS) rRNA gene amplicon sequencing. Finally, we compared the intermicrobial correlations in patients with UC and non-IBD controls.

MATERIALS AND METHODS

Patient selection and stratification

Patients with UC and non-IBD controls were recruited from patients admitted to Peking Union Medical College Hospital in Beijing, China from January 1, 2018 to June 30, 2019. The diagnosis of UC was determined by endoscopic and pathological findings. Only patients with regular follow-ups (more than 3 times in 1 year, outpatient and/or inpatient) were included, and all these patients orally took 1 g of mesalazine per time, 3-4 times per day. Patients with any history of colectomy, antibiotic use or probiotic application within 3 mo before sample collection, or other types of gastroenteric complications (including gastrointestinal tumor, gastroduodenal ulcer, and irritable bowel syndrome) were excluded. The clinical activity of disease was determined by the Mayo score/Disease Activity Index[21], and the patients were classified into UC in remission or active UC groups, with the latter further divided into mild, moderate, and severe groups. The patients were also classified into proctitis (type E1), distal colitis (type E2), or pancolitis (type E3) groups based on their disease types according to the Montreal classification[22]. The inflammatory status of the biopsy was confirmed by pathological evidence. The non-IBD controls were asymptomatic patients with colonic polyps.

This work was been approved by the ethical committees of Peking Union Medical College Hospital (No. JS-1488). All the patients included have signed an informed consent form.

Sample collection

Fecal samples were collected 1-2 d before bowel preparation and endoscopic examination, and were stored in stool tubes (Allwegene Company, Beijing) at -80 °C within 2 h after collection. Two biopsies were taken from each biopsy site during endoscopy. One of the biopsies was stored in formalin for standard histological staining, and the other was snap-frozen and then stored at -80 °C for sequencing.

DNA extraction and polymerase chain reaction amplification

DNA was extracted from samples using the DNeasy PowerSoil Kit (QIAGEN, Dusseldorf, GER) according to the manufacturer's protocol. The purity and quality of the genomic DNA were checked on 0.8% agarose gels. The V3-4 hypervariable region of the bacterial 16S rRNA gene was amplified with the primers 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT)[23]. The fungal ITS gene was amplified with the primers ITS3-F (GCATCGATGAAGAACGCAGC) and ITS4-R (TCCTCCGCTTATTGATATGC)[24]. For each mucosa or fecal sample, a 10-digit barcode sequence was added to the 5' end of the forward and reverse primers. Polymerase chain reaction (PCR) was carried out on a Mastercycler Gradient (Eppendorf, Germany) using a 25 μL reaction volume containing 12.5 μL of 2 × Taq PCR MasterMix, 3 μL of BSA (2 ng/μL), primers (5 μM), 2 μL of template DNA, and 5.5 μL of ddH_2O. The cycling parameters were 95 °C for 5 min, followed by 32 cycles of 95 °C for 45 s, 55 °C for 50 s, and 72 °C for 45 s with a final extension at 72 °C for 10 min. Three PCR products per sample were pooled to mitigate reaction-level PCR biases. The PCR products were purified using a QIAquick Gel Extraction Kit (QIAGEN, Germany), quantified using real-time PCR, and sequenced by Allwegene

Company (Beijing, China).

High-throughput sequencing and comparative sequence analysis

Deep sequencing was performed on the Miseq platform at Allwegene Company (Beijing). After the run, image analysis, base calling, and error estimation were performed using Illumina Analysis Pipeline Version 2.6. The raw data were first screened, and sequences were removed if they were shorter than 150 bp or longer than 500 bp, had a low-quality score (≤ 20), contained ambiguous bases, or did not exactly match the primer sequences and barcode tags. After screening, the qualified reads were separated using the sample-specific barcode sequences and trimmed with Illumina Analysis Pipeline Version 2.6. Then, the dataset was analyzed using Quantitative Insights Into Microbial Ecology (QIIME, Ver2.1, Rob Knight laboratory and Greg Caporaso laboratory, United States). After 16S rRNA amplicon sequencing and screening, an average of 59307 ± 4058 [mean ± standard error of mean (SEM)] qualified reads were obtained from the fecal samples, and an average of 54201 ± 3834 qualified reads were obtained from the mucosa samples. After ITS sequencing and screening, an average of 47051 ± 4675 qualified reads were obtained from the fecal samples. In the subsequent subgroup analysis, singletons were further removed from the qualified sequences and then the latter was subsampled and clustered into operational taxonomic units (OTUs) at a similarity level of 97% to generate rarefaction curves and to calculate the richness and diversity indices (the number of subsampled sequences and the corresponding OTUs for each subgroup analysis are described in Supplementary Table 1. The Ribosomal Database Project Classifier tool was used to classify all sequences into different taxonomic groups. Finally, the bacterial taxonomic groups were matched to specific genera based on Silva database (Ver. 128), and the fungal groups were based on the Unite database (Ver. 7.1).

Statistical analysis

A *t*-test was used to detect the difference in age among groups. The chi-square test was used to compare the distributions of sex, disease type, and disease activity among groups. The alpha diversity, principal component analysis (PCA), and analysis of similarities (ANOSIM) were performed based on the OTUs and the abundance acquired from the subsampled sequences of samples involved in the subgroup analysis. Alpha diversity was estimated using Shannon index metric, and one-way ANOVA (SPSS 19.0) was used to measure the difference among Shannon indices. To examine the similarity of the microbiota structure between different samples, PCA was performed using R software. PCA was used to reduce the high dimensionality of the intestinal microbiota structure, which contained numerous original variables, and to observe the relationship among samples in a rectangular coordinate. To further examine the similarity between different samples and to determine whether the between-group difference was greater than the inter-group difference, ANOSIM was performed in the R. The Metastats analysis using Mothur was employed to detect interindividual differences and the differences in microbiota composition among groups at the phylum and genus levels were measured. Only bacteria or fungi with relative abundances higher than 1% in either group were displayed in this study and were discussed with emphasis. Generally, the numerical value is shown as the mean ± SD. Under the condition of multiple comparisons, the *P* values were corrected to control for the false discovery rate by Benjamini-Hochberg method ($\alpha = 0.05$). Differences with a $P < 0.05$ were considered statistically significant. Moreover, Spearman correlation coefficient analysis was applied and inter-microbial correlations at the genus level with a *P* value less than 0.05 and an R value greater than 0.6 were selected and put into a correlation network using Cytoscape v3.7.0. The statistical methods of this study were reviewed by Dr. Tao-Tao Han from Chinese Academy of Medical Sciences and Peking Union Medical College.

RESULTS

Sample collection and microbiota analysis

Eighty-nine patients with UC and 33 non-IBD controls were included in the present study. Among the 89 patients with UC, 31 provided both stool and mucosa samples and 19 patients with UC at active stage provided mucosa samples from inflamed and noninflamed sites, and finally 72 stool samples and 67 mucosa samples were obtained. Twenty-one stool samples and 12 mucosa samples were obtained from non-IBD controls diagnosed with tubular adenoma, tubulovillous adenoma, or a hyperplastic

polyp according to histological evidence. The basic clinical characteristics of the subjects among the groups under different stratifications were compared (Supplementary Tables 2-4), including age, sex proportion, disease activity, and disease type distribution in patients with UC. Most of the characteristics showed little intergroup difference, except for the mean age of the non-IBD controls and patients with UC and the disease type distribution of active UC and UC in remission. Among the stool samples, the compositions of fungal flora in 15 non-IBD samples and 42 UC samples were detected by ITS sequencing. The rest of the samples (6 non-IBD controls and 30 UC patients) failed to undergo ITS sequencing due to the insufficiency of tissues. Although a difference existed in the results of OTUs clustering in different subgroup analysis, in both the fecal and the mucosa sample, *Firmicutes*, *Bacteroidetes*, *Proteo-bacteria*, and *Actinobacteria* were always the four chief components among the identified phyla, and the identified fecal fungi mainly consisted of *Basidiomycota* and *Ascomycota*.

Fecal and mucosal bacterial microbiota of patients with UC is different

The microbiota in the stool and mucosa samples of the same patient with UC were compared if collected within less than 3 d, and 31 pairs of samples were included. Compared with the mucosal sample (Shannon index, 5.04 ± 1.14), the α-diversity of the microbiota in the fecal sample (Shannon index, 4.08 ± 0.89) was significantly lower (P = 0.001) (Figure 1A). As directly assessed by PCA (Figure 1B), the flora structure of the mucosa sample and that of the stool sample showed obvious differences and ANOSIM indicated significant intergroup differences (Figure 1C). The microbiota compositions at the phylum and genus levels of the mucosa and fecal samples are shown in Figure 1D. At the phylum level, the relative abundance of mucosal *Proteobacteria* and unidentified phyla was higher than that of fecal *Proteobacteria*, while the relative abundances of *Bacteroidetes* and *Firmicutes* were lower in the mucosa sample. At the genus level, 16 types of bacterial genera differed significantly between the mucosa and stool samples (the relative abundance and the P value of each bacterium are described in Supplementary Table 5).

Comparison between patients with UC with different levels of disease activity and non-IBD controls–for stool samples

The α-diversity of intestinal bacteria decreases in patients with UC and the microbiota composition differs among UC patients in remission, patients with active UC, and non-IBD controls: As shown in Figure 2A, compared with the non-IBD controls (Shannon index, 4.71 ± 0.64), the α-diversity of the intestinal microbiota evaluated by the Shannon index significantly decreased in patients with UC in remission (Shannon index, 4.11 ± 0.74; P = 0.013), as well as in patients with active UC (Shannon index, 3.20 ± 1.04; P = 0.000). The α-diversity of patients with active UC was also significantly lower than that of UC patients in remission (P = 0.002). According to the PCA and ANOSIM results, the intergroup difference between the non-IBD controls and UC patients in remission group was significant (Figure 2B and C). The microbiota compositions of the different groups are shown in Figure 2D (the relative abundance and P value are listed in Supplementary Table 6). At the phylum level, the abundance of *Proteobacteria* significantly increased in patients with active UC but decreased in patients with UC in remission, while that of *Firmicutes* showed the opposite pattern. The abundance of *Bacteroidetes* significantly decreased in both the active UC and UC in remission groups. At the genus level, compared with the non-IBD controls, the relative abundances of *Alistipes*, *Bacteroides*, *Dialister*, and *Escherichia-Shigella* in patients with UC in remission significantly decreased, while that of *Lachnospira* increased. The relative abundances of *Escherichia-Shigella*, *Enterococcus*, and *Peptoclostridium* were significantly higher in the active UC group than in the non-IBD group, while those of *Alistipes*, *Subdoligranulum*, *Roseburia*, *Ruminococcus* 2, and *Ruminococcus torques* were significantly lower in patients with active UC. When comparing patients with active UC and UC in remission, the relative abundances of *Escherichia-Shigella*, *Enterococcus*, *Haemophilus*, and *Klebsiella* were higher in the former, while those of *Lachnospira*, *Faecalibacterium*, *Roseburia*, and *Blautia* were higher in the latter.

The α-diversity continuously decreases, and some bacteria show a consistently changing pattern with increasing disease activity in patients with active UC: As shown in Figure 3A, the α-diversity of the intestinal microbiota gradually decreased with increasing inflammation degree (the Shannon index of mild, moderate, and severe active UC was 3.74 ± 0.84, 3.08 ± 0.95, and 2.66 ± 1.14, respectively), and the difference between the mild and moderate groups (P = 0.025), and between the mild and severe groups (P = 0.003) was significant, though that between the moderate and

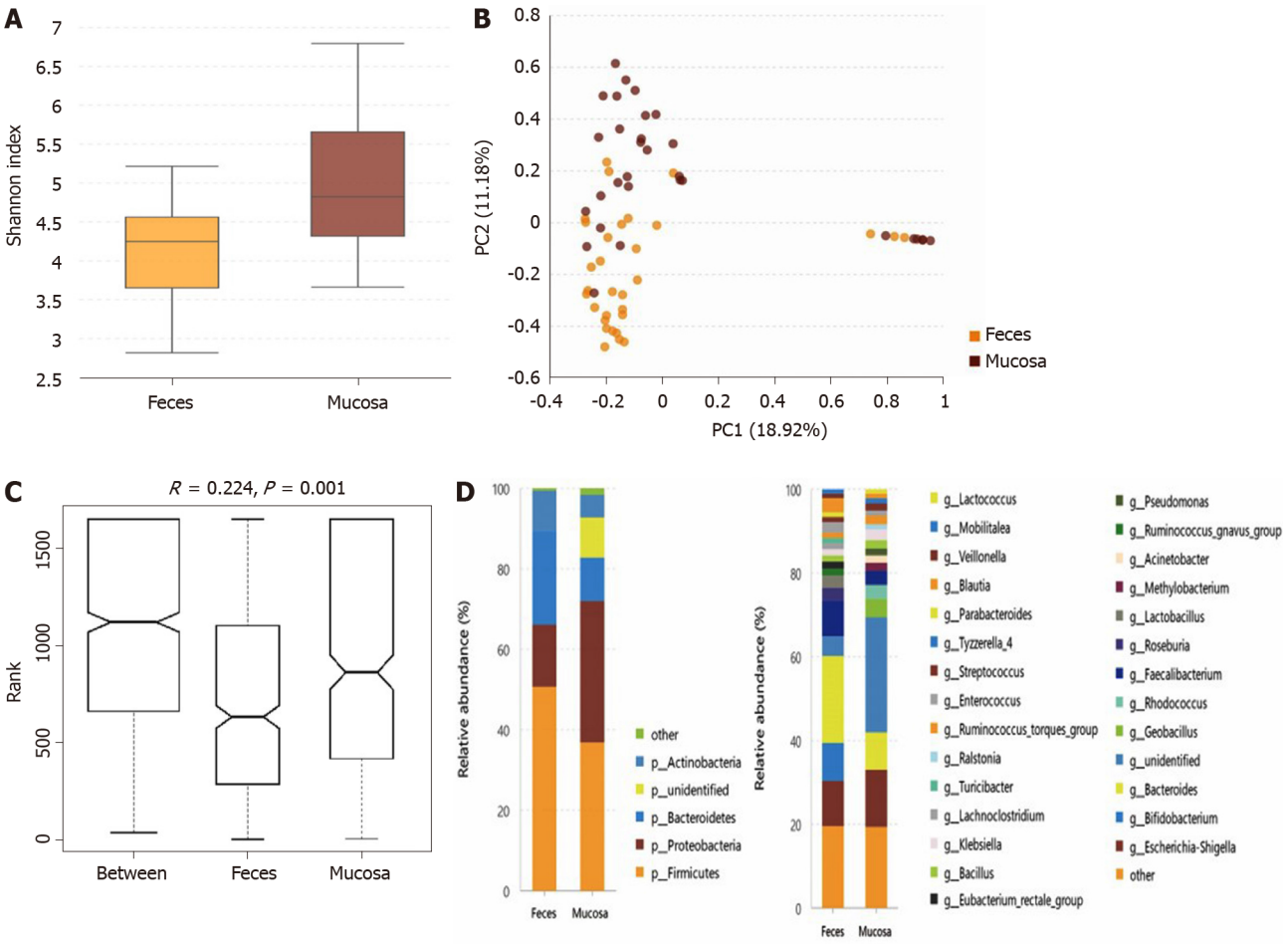


Figure 1 tatistical analysis of the sequencing results of the bacteria microbiota in stool and mucosa samples from patients with ulcerative colitis. A: The α-diversity of microbiota evaluated by Shannon index. The ordinate shows the Shannon index, and the abscissa shows the sample types. Compared with mucosa samples (Shannon index, 5.04 ± 1.14), the α-diversity of microbiota in fecal samples (Shannon index, 4.08 ± 0.89) was significantly lower (P = 0.001); B: Principal component analysis. The flora structure of mucosa samples and that of stool samples showed an obvious difference (with a PC1 of 18.92% and a PC2 of 11.18%, as showed in the abscissa and ordinate axis separately); C: The analysis of similarities. The intergroup difference (marked as Between) was greater than within group differences (marked as Feces and Mucosa for the two groups) and the result was significant (P = 0.001); D: The relative abundance of the bacteria at the phylum (the left bar graph) and genus (the right bar graph) levels. The ordinate shows the relative abundance (%), and the abscissa axis shows the sample types where the microbiota was sequenced from. At the phylum level, the relative abundance of mucosal *Proteobacteria* and the unidentified phyla was higher than fecal ones, while the relative abundance of *Bacteroidetes* and *Firmicutes* was lower in the mucosa samples. At the genus level, 16 kinds of bacteria genera differed significantly between mucosa and stool samples.

severe groups was not obvious (P = 0.235). Significant differences were observed through PCA (Figure 3B) and ANOSIM (Figure 3C). The microbiota compositions of different groups are shown in Figure 3D (the relative abundance and P value are listed in Supplementary Table 7). At the phylum level, the abundance of *Proteobacteria* showed a significantly increasing trend with deteriorating disease conditions, and that of *Bacteroidetes* gradually decreased, although only the difference between the mild and severe groups was obvious. At the genus level, some bacterial genera that differed significantly between the non-IBD controls and patients with active UC displayed consistently changing patterns when the disease condition worsened, including a further increase in *Escherichia-Shigella* in moderate-severe active UC and an obvious reduction of *Faecalibacterium*, *Roseburia*, and *Bacteroides* in severe active UC. The relative abundance of *Eubacterium rectale* group gradually decreased when the inflammation worsened, although this genus did not differ significantly between the non-IBD controls and patients with UC. Decreased *Blautia* in moderate active UC and reduced *Parasutterella* and *Lachnospira* in severe active UC compared with the mild UC group were also observed.

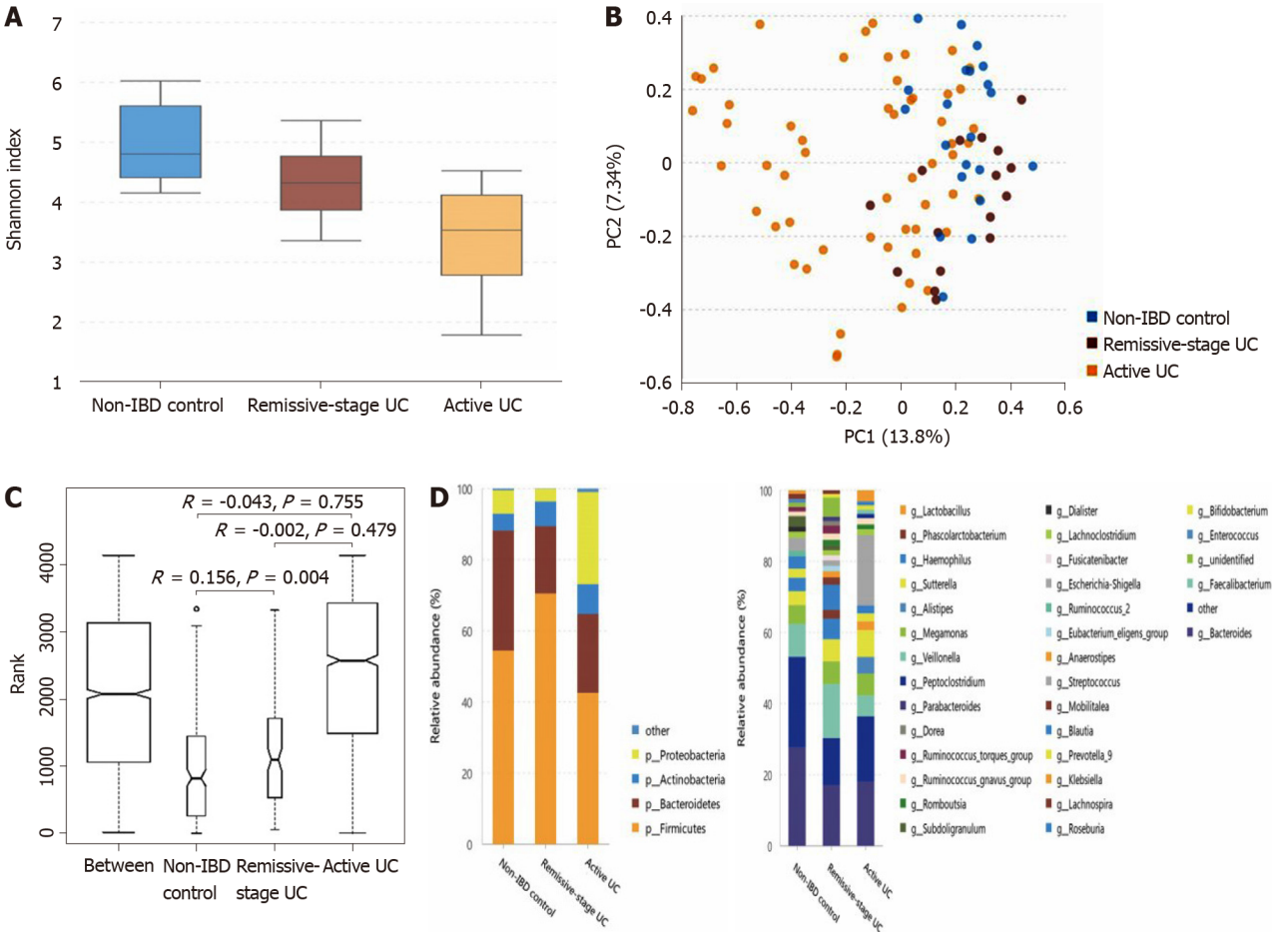


Figure 2 Statistical analysis of the sequencing results of the bacteria microbiota in stool samples from patients with ulcerative colitis and non-inflammatory bowel disease controls. A: The α-diversity of microbiota evaluated by Shannon index. The ordinate shows the Shannon index, and the abscissa shows the groups. Compared with non-inflammatory bowel disease (IBD) controls (Shannon index, 4.71 ± 0.64), the α-diversity of intestinal microbiota evaluated by Shannon index significantly decreased in the ulcerative colitis (UC) in remission (Shannon index, 4.11 ± 0.74; P = 0.013), as well as in the active patients with UC (Shannon index, 3.20 ± 1.04; P = 0.000). The α-diversity of active UC was also significantly lower than that of UC in remission (P = 0.002); B: Principal component analysis. The flora structure of non-IBD controls and UC in remission group showed an obvious difference (with a PC1 of 13.8% and a PC2 of 7.34%, as showed in the abscissa and ordinate axis separately); C: The analysis of similarities. The intergroup difference between non-IBD controls and UC in remission group was greater than within group differences, and the result was significant (P = 0.004); D: The relative abundance of the bacteria at the phylum (the left bar graph) and genus (the right bar graph) levels. The ordinate shows the relative abundance (%), and the abscissa axis shows the sample types where the microbiota was sequenced from. At the phylum level, the abundance of *Proteobacteria* significantly increased in active UC but decreased in UC in remission, while that of *Firmicutes* showed the opposite pattern. The abundance of *Bacteroidetes* significantly decreased in both active UC and UC in remission groups. At the genus level, compared with non-IBD controls, the relative abundance of *Alistipes*, *Bacteroides*, *Dialister*, and *Escherichia-Shigella* in UC in remission significantly decreased, while that of *Lachnospira* increased. The relative abundance of *Escherichia-Shigella*, *Enterococcus*, and *Peptoclostridium* was significantly higher in active UC group than in the non-IBD group, while that of *Alistipes*, *Subdoligranulum*, *Roseburia*, *Ruminococcus 2*, and *Ruminococcus torques* group was significantly lower in active patients with UC. When comparing active UC and UC in remission, the relative abundance of *Escherichia-Shigella*, *Enterococcus*, *Haemophilus*, and *Klebsiella* was higher in the former, while that of *Lachnospira, Faecalibacterium*, *Roseburia*, and *Blautia* was higher in the latter. IBD: Inflammatory bowel disease; UC: Ulcerative colitis.

Comparison between patients with UC with different levels of disease activity and non-IBD controls–for mucosa samples

The α-diversity of the mucosal microbiota decreases in patients with active UC, and the microbiota composition differs among groups: As shown in Figure 4A, the α-diversity of the mucosal microbiota in the non-IBD controls (Shannon index, 6.12 ± 0.70) was similar to that in patients with UC in remission (Shannon index, 5.63 ± 1.53; P = 0.682) but was significantly higher than that in patients with active UC (4.99 ± 1.02; P = 0.001). The α-diversity of patients with UC in remission and with active UC showed little difference (P = 0.453). Direct observation of the PCA rectangular coordinates (Figure 4B) indicated that the flora structure of the non-IBD controls was different from that of either UC group. ANOSIM indicated that the difference between the controls and patients with UC in remission was significant (Figure 4C). As shown in Figure 4D (the relative abundance and P value are listed in Supplementary Table 6), at the phylum level, compared with the non-IBD controls, the relative abundances of

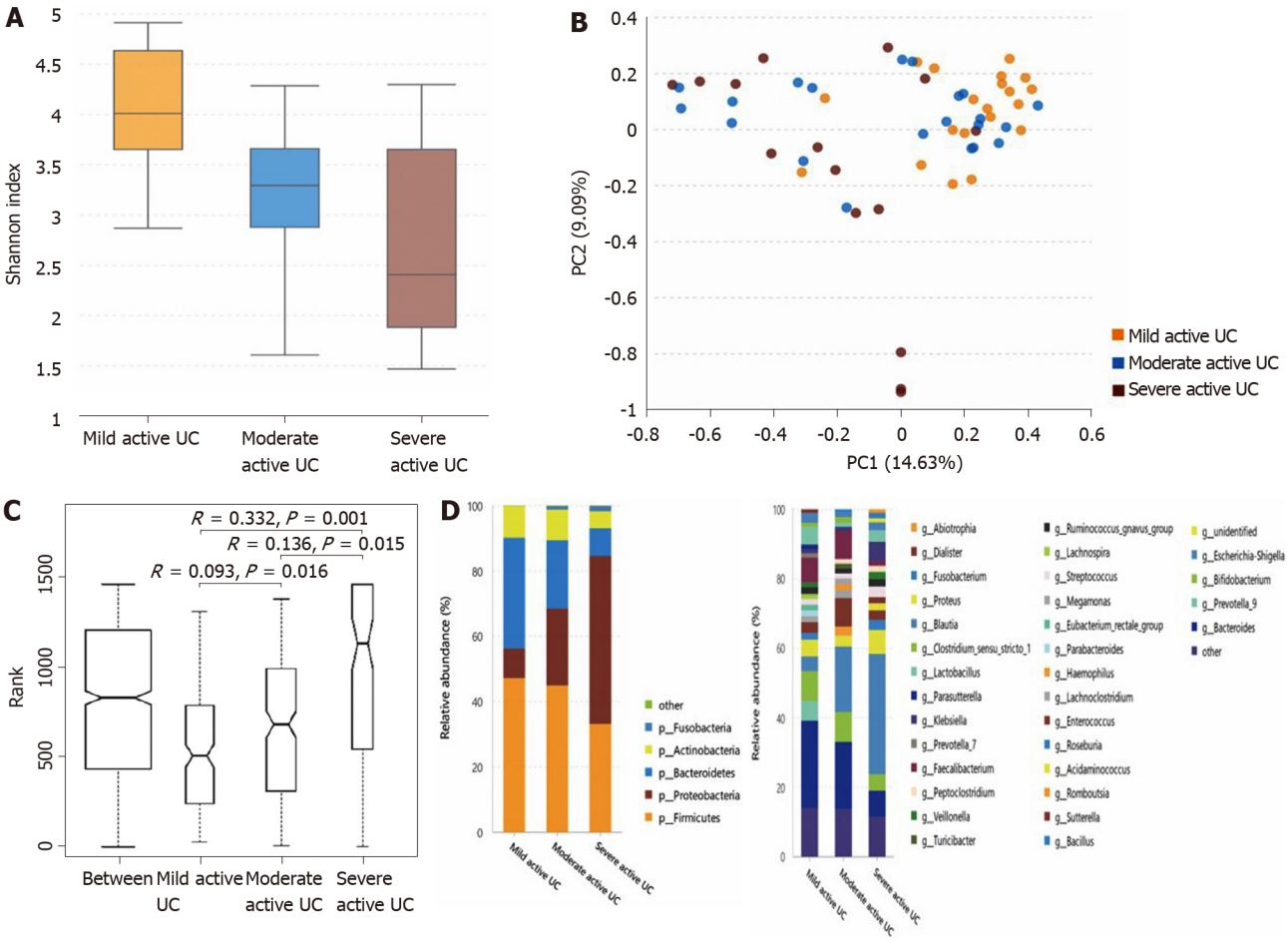


Figure 3 Statistical analysis of the sequencing results of the bacteria microbiota in stool samples from mild, moderate, and severe active ulcerative colitis groups. A: The α-diversity of microbiota evaluated by Shannon index. The ordinate shows the Shannon index, and the abscissa shows the groups. The α-diversity of the intestinal microbiota gradually decreased along with increasing inflammation degree (the Shannon index of mild, moderate, and severe active ulcerative colitis (UC) was 3.74 ± 0.84, 3.08 ± 0.95, and 2.66 ± 1.14, respectively), and the difference between the mild and moderate groups (P = 0.025), and between the mild and severe groups (P = 0.003) was significant, although that between it was not significantly between the moderate and severe groups (P = 0.235); B: Principal component analysis. The flora structure of mild, moderate, and severe active UC groups showed an obvious difference (with a PC1 of 14.63% and a PC2 of 9.09%, as showed in the abscissa and ordinate axis separately); C: The analysis of similarities. The intergroup differences between any two of these groups were significant (P = 0.016 between the mild and the moderate stage group, P = 0.015 between the moderate and the severe stage group, and P = 0.001 between the mild and the severe stage group); D: The relative abundance of the bacteria at the phylum (the left bar graph) and genus (the right bar graph) levels. The ordinate shows the relative abundance (%), and the abscissa axis shows the sample types where the microbiota was sequenced from. At the phylum level, the abundance of *Proteobacteria* showed a significant trend of increase with a deteriorating disease condition, and that of *Bacteroidetes* gradually decreased, though only the difference between mild and severe subgroups was obvious. At the genus level, some bacteria genera that differed significantly between non-inflammatory bowel disease (IBD) controls and active patients with UC displayed a consistent changing pattern when the disease condition was getting worse, including a further increase of *Escherichia-Shigella* in moderate-severe active UC and an obvious reduction of *Faecalibacterium*, *Roseburia*, and *Bacteroides* in severe active UC. The relative abundance of *Eubacterium rectale* group gradually decreased when the inflammation worsened though this genus did not differ significantly between non-IBD controls and patients with UC. Decreased *Blautia* in moderate active UC and reduced *Parasutterella* and *Lachnospira* in severe active UC compared with the mild group were also observed. UC: Ulcerative colitis.

Firmicutes and *Proteobacteria* significantly increased in patients with active UC, and that of *Actinobacteria* was significantly higher in patients with both active UC and UC in remission. The relative abundance of unidentified bacterial phyla obviously decreased in patients with UC in remission and further decreased in patients with active UC. At the genus level, the relative abundances of *Geobacillus*, *Lactococcus*, *Pseudomonas*, *Methylobacterium*, *Acinetobacter*, *Streptococcus*, *Bacillus*, and *Ralstonia* increased in the active UC group compared with the non-IBD controls, while those of *Ruminococcus torques* and the unidentified bacterial genera were reduced. When comparing the non-IBD controls and patients with UC in remission, the relative abundances of *Eubacterium rectale* and the unidentified genera were lower in the latter, while those of *Methylobacterium*, *Rhodococcus*, *Peptoclostridium*, and *Faecalibacterium* were higher. The difference between patients with active UC and UC in remission was also obvious, and more *Geobacillus*, *Lactococcus*, *Pseudomonas*, *Streptococcus*, *Bacillus*, *Ralstonia*, and *Prevotella group 9* were observed in patients with active UC.

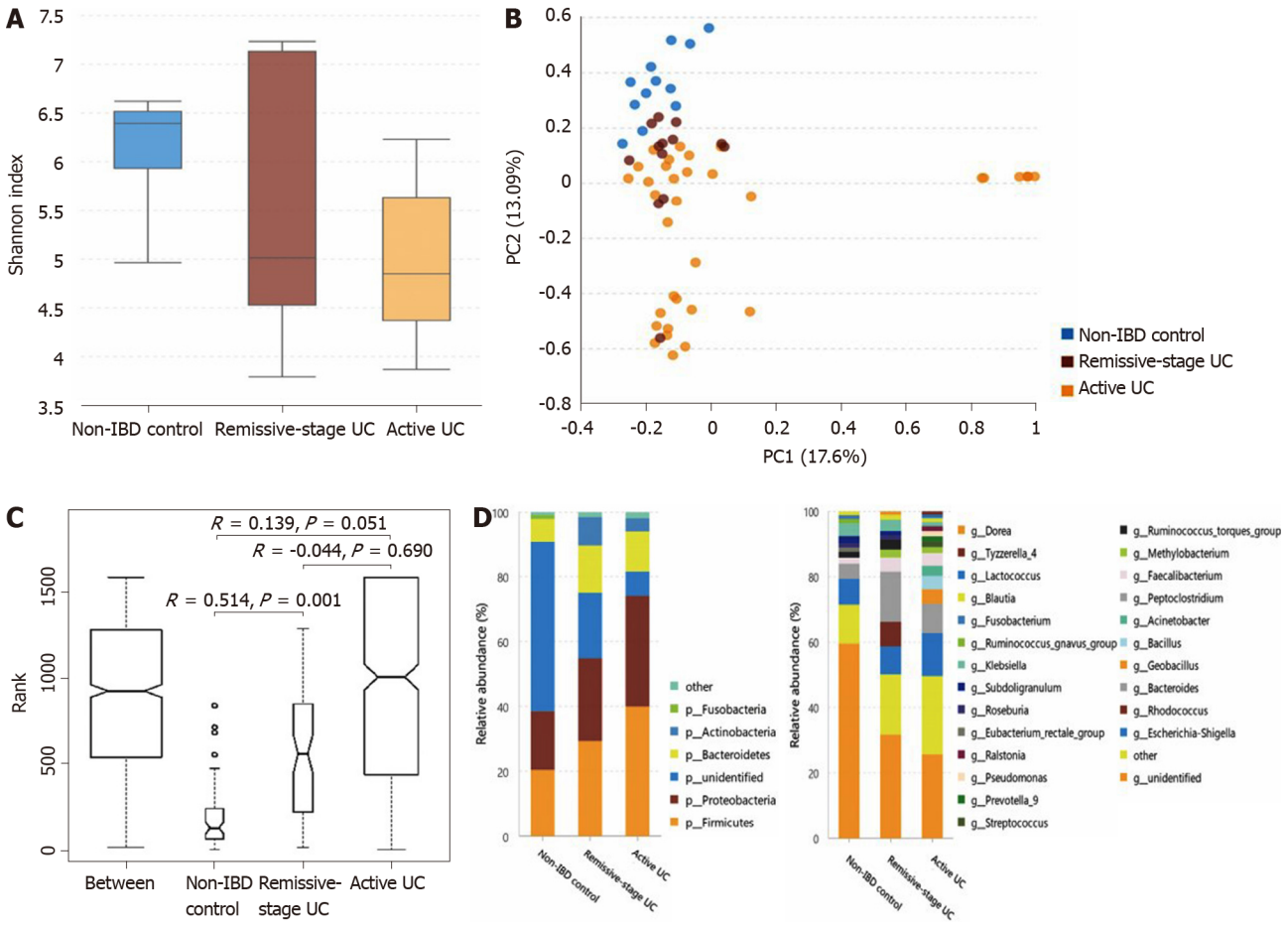


Figure 4 Statistical analysis of the sequencing results of the bacteria microbiota in mucosa samples from patients with ulcerative colitis and non-inflammatory bowel disease controls. A: The α-diversity of microbiota evaluated by Shannon index. The ordinate shows the Shannon index, and the abscissa shows the groups. The α-diversity of mucosal microbiota in non-inflammatory bowel disease (IBD) controls (Shannon index, 6.12 ± 0.70) was similar to that in ulcerative colitis (UC) in remission (Shannon index, 5.63 ± 1.53; P = 0.682) but significantly higher than that of the active UC group (4.99 ± 1.02; P = 0.001). And the α-diversity of UC in remission and active UC showed little difference (P = 0.453); B: Principal component analysis. The flora structure of non-IBD controls was different from that of either UC group (with a PC1 of 17.6% and a PC2 of 13.09%, as showed in the abscissa and ordinate axis separately); C: The analysis of similarities. The intergroup difference between controls and UC in remission was significant (P = 0.001); D: The relative abundance of the bacteria at the phylum (the left bar graph) and genus (the right bar graph) levels. The ordinate shows the relative abundance (%), and the abscissa axis shows the sample types where the microbiota was sequenced from. At the phylum level, compared with non-IBD controls, the relative abundance of *Firmicutes* and *Proteobacteria* significantly increased in active UC, and that of *Actinobacteria* was significantly higher in both the active UC and UC in remission groups. The relative abundance of unidentified bacterial phyla obviously decreased in UC in remission and further decreased in active UC. At the genus level, the relative abundance of *Geobacillus*, *Lactococcus*, *Pseudomonas*, *Methylobacterium*, *Acinetobacter*, *Streptococcus*, *Bacillus*, and *Ralstonia* increased in active UC compared with non-IBD controls, while that of *Ruminococcus torques* group and unidentified bacteria genera decreased. When comparing the non-IBD controls and UC in remission, the relative abundance of *Eubacterium rectale* group and unidentified genera was lower in the latter while that of *Methylobacterium*, *Rhodococcus*, *Peptoclostridium*, and *Faecalibacterium* was higher. The difference between active UC and UC in remission was also obvious and more *Geobacillus*, *Lactococcus*, *Pseudomonas*, *Streptococcus*, *Bacillus*, *Ralstonia*, and *Prevotella* group 9 were observed in active patients with UC. IBD: Inflammatory bowel disease; UC: Ulcerative colitis.

The α-diversity of mucosal bacteria further decreases in severe active UC, and the microbiota composition is slightly different among subgroups that vary in inflammation degree: The α-diversity of mucosal bacteria in patients with severe active UC (Shannon index, 3.67 ± 1.25) was significantly lower than that in patients with mild active UC (Shannon index, 5.40 ± 1.01; P = 0.017). However, little difference was observed between the mild and moderate subgroups (Shannon index, 4.80 ± 0.90; P = 0.100) or between the moderate and severe subgroups (P = 0.251) (Figure 5A). Only the flora structure of the mild and severe groups showed obvious differences according to PCA and ANOSIM (Figure 5B and C). As shown in Figure 5D (the relative abundance and P value are listed in Supplementary Table 7), at the phylum level, the relative abundance of *Actinobacteria* in patients with severe active UC was significantly lower than that in patients with mild/moderate UC, and fewer unidentified bacterial phyla were observed in patients with severe UC than in patients with mild UC. At the genus level, the relative abundance of *Methylobacterium* in patients with mild UC was significantly higher than that in patients with moderate/severe UC, and *Blautia*

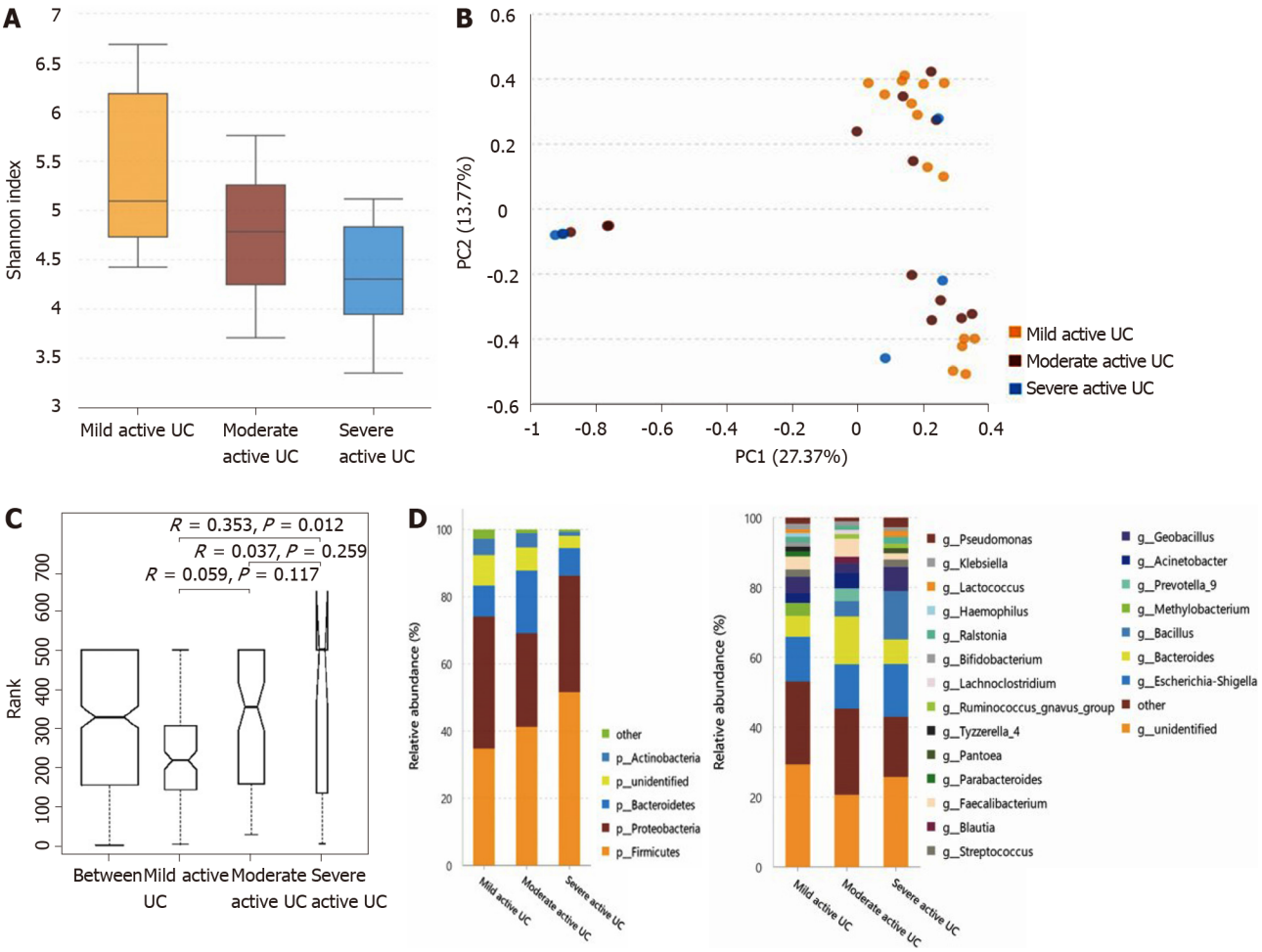


Figure 5 Statistical analysis of the sequencing results of the bacteria microbiota in mucosa samples from mild, moderate, and severe active ulcerative colitis groups. A: The α-diversity of microbiota evaluated by Shannon index. The ordinate shows the Shannon index, and the abscissa shows the groups. The α-diversity of mucosal bacteria in severe active ulcerative colitis (UC) (Shannon index, 3.67 ± 1.25) was significantly lower than that of mild active UC (Shannon index, 5.40 ± 1.01; $P = 0.017$). Little difference was observed between the mild and moderate groups (Shannon index 4.80 ± 0.90; $P = 0.100$), neither between the moderate and severe ones ($P = 0.251$); B: Principal component analysis. The flora structure of mild and severe subgroups showed a significant difference (with a PC1 of 27.37% and a PC2 of 13.77%, as showed in the abscissa and ordinate axis separately); C: The analysis of similarities. The intergroup difference between mild and severe groups was significant ($P = 0.012$); D: The relative abundance of the bacteria at the phylum (the left bar graph) and genus (the right bar graph) levels. The ordinate shows the relative abundance (%), and the abscissa axis shows the sample types where the microbiota was sequenced from. At the phylum level, the relative abundance of *Actinobacteria* of severe active UC was significantly lower than that of mild/moderate UC, and fewer unidentified bacteria phyla were observed in severe UC than in mild UC. At the genus level, the relative abundance of *Methylobacterium* in mild UC was significantly higher than that in moderate/severe UC, and *Blautia* significantly decreased in severe UC compared with the other two groups. UC: Ulcerative colitis.

significantly decreased in patients with severe UC compared with the other two subgroups.

Comparison between intestinal bacteria in patients with active UC with different disease extents or different biopsy sites

Patients with active UC with different disease extents show slight differences in bacterial α-diversity and composition: Based on the extent of colitis, patients with active UC were divided into two groups: Partial colitis (E1 and E2) and pancolitis groups (E3). The α-diversity of fecal bacteria in these groups was similar, while that of mucosal bacteria was higher in the pancolitis group. Regarding the bacterial composition in stool samples, only the relative abundance of the phylum *Fusobacteria* and its genus *Fusobacterium* was significantly higher in the stool of pancolitis patients. Regarding the mucosa sample, the relative abundance of unidentified phyla and the genus *Methylobacterium* in the "partial colitis" group was significantly lower than that in the pancolitis group (Supplementary Figures 1 and 2, and Supplementary Table 8).

Mucosa samples from inflamed and noninflamed sites have similar intestinal microbiota structures: Nineteen pairs of mucosa samples from inflamed and

noninflamed sites of one subject were collected. There was no significant difference between the microbiota of mucosa samples from the inflamed and noninflamed sites as evaluated by the α-diversity, PCA, ANOSIM, or microbiota composition at the phylum and genus levels (Supplementary Figure 3).

Analysis of fecal fungi and bacteria-fungi correlations

The α-diversity and composition of stool fungal microbiota slightly differ among groups: There was little difference between the fecal fungal microbiota of patients with UC and the non-IBD controls or among the active UC groups with different degrees of inflammation as evaluated by α-diversity, PCA, or ANOSIM. Regarding the fungal composition, only a few fungal genera differed significantly, including a higher relative abundance of *Mycosphaerella*, *Malassezia*, and/or *Suillus* in patients with active UC, more *Xeromyces* and *Yarrowia* in patients with severe active UC, and more *Rhizopus* in patients with moderate active UC (Supplementary Figures 4 and 5, and Supplementary Table 9).

The intestinal intermicrobial correlation changes in patients with UC compared with non-IBD controls: The intestinal intermicrobial correlations at the genus level in the non-IBD controls and the UC in remission and active UC groups are shown in a correlation network (Figure 6). In all of the groups, most of the bacteria-bacteria correlations existed in *Firmicutes*, the majority of the bacteria-fungi correlations occurred in *Firmicutes* and *Ascomycota*, and the *Ascomycota-Ascomycota/Basidiomycota* correlations were the major fungi-fungi correlations. Compared with the controls, the number of significant correlations increased in patients with UC in remission but decreased in patients with active UC. The most obvious change occurred in the number of bacteria-fungi correlations, while the number of fungi-fungi correlations was insignificant among the three groups.

To further investigate the change in bacteria-fungi correlations, we focused on the bacterium-fungus pairs in which at least one of the two genera had a relative abundance higher than 0.01, and the correlations with a *P* value smaller than 0.05 are shown in the heat map (Supplementary Figure 6). In the non-IBD controls, bacterial genera from nine bacterial phyla were correlated with fungal genera from four fungal phyla, while for patients with UC in remission, the number of involved bacterial phyla decreased to five, and for patients with active UC, the number of phyla was further reduced to three. In addition, the correlation intensity of the related bacterium-fungus pairs was distributed evenly among the controls, while the correlation intensity of some microbe pairs in patients with UC became prominent, indicating that the bacteria-fungi interaction became single and circumscribed in patients with UC.

DISCUSSION

In this study, the intestinal bacteria flora of patients with UC was analyzed, and three important results were acquired. First, the fecal bacteria and mucosal bacteria of patients with UC were different, and the former was characterized by a lower α-diversity, lower relative abundances of *Proteobacteria* and unidentified phyla, and higher proportions of *Bacteroidetes* and *Firmicutes*. Significant differences were also shown in 16 bacterial genera. This result agrees with a previous study showing that the sample origin (biopsy *vs* stool) is the single most influential factor on the microbial community structure in IBD patients[10]. Another study also reported fecal-specific bacterial species[11]. Bowel preparation impacted the mucosal microbiota α-diversity (mainly presented as decreased diversity) and the abundance of certain bacteria[25], which may partially contribute to the difference between biopsy and fecal samples. The vastly distinct environment for the colonization of microbes may be the principal factor.

Second, obvious distinctions were observed between the non-IBD controls and patients with UC. Notably, the intestinal bacteria in patients with UC in remission and active UC may be regarded as two dissimilar populations owing to their significantly different α-diversity and flora structure. The former was more similar to that of the non-IBD controls, as reflected by a smaller decrease in α-diversity and smaller changes in the bacterial composition. Moreover, the bacterial composition of the active and relieved UC groups even showed opposite changing patterns in some circumstances; for example, *Proteobacteria* and its genus *Escherichia-Shigella* increased in the feces of active UC but decreased in UC in remission, while *Firmicutes* and some of its members changed oppositely. Previous studies have confirmed a depletion of α-diversity in

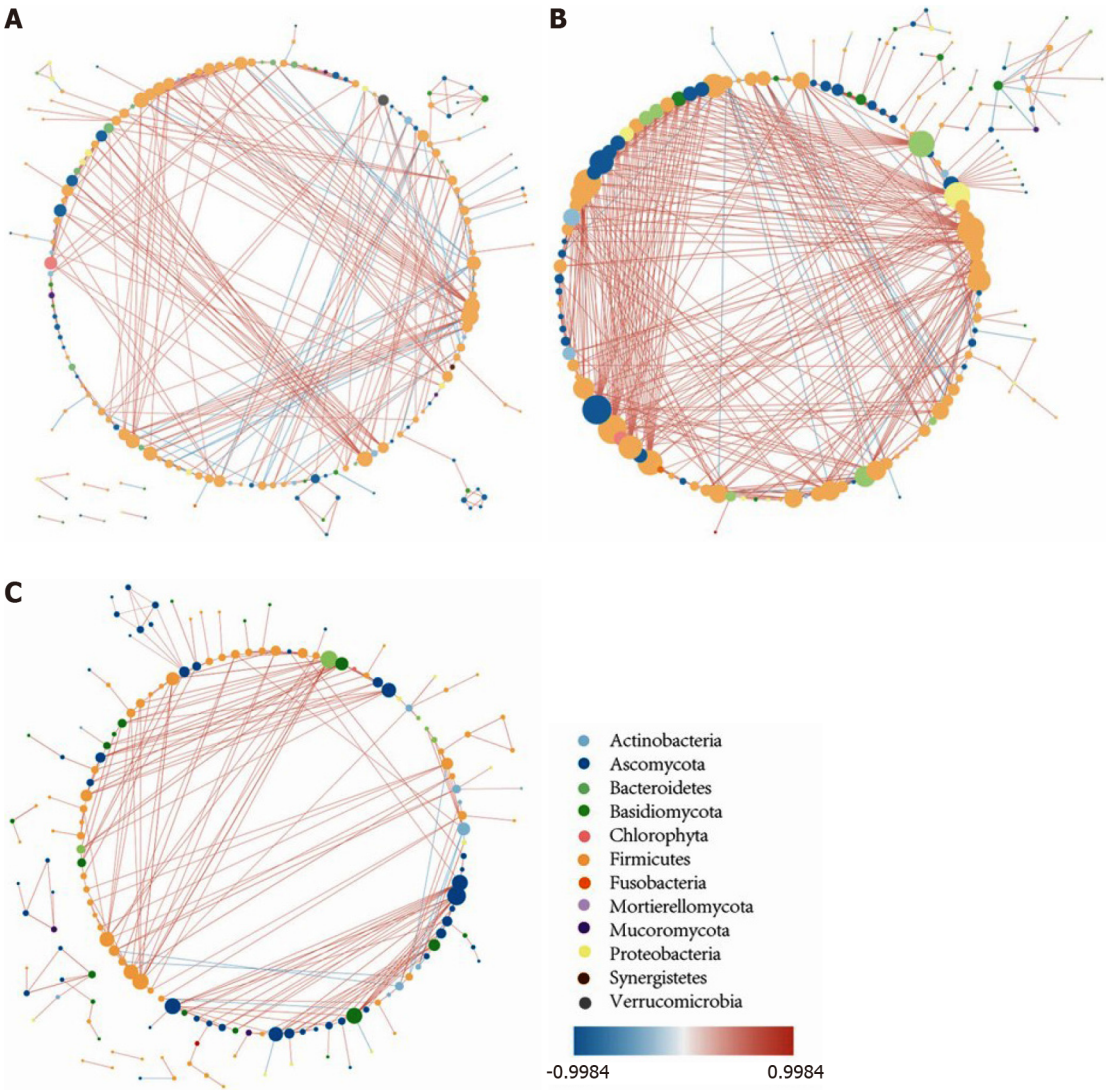


Figure 6 The intestinal microbiota correlation network. A: The intestinal microbiota correlation network of non-inflammatory bowel disease controls; B: The intestinal microbiota correlation network of ulcerative colitis (UC) in remission; C: The intestinal microbiota correlation network of active UC. The network was constructed based on Spearman correlation coefficient analysis and the software Cytoscape v3.7.0., and only the significant correlations are shown ($P < 0.05$). Each node represents one microbial genus and its color represents the phylum that it belongs to. The size of the node is equal to the number of correlated pairs that it evolves. The depth of color of the line connecting two nodes reveals the strength of the association. The red color indicates positive correlations and the blue color indicates negative correlations. Compared with controls, the number of significant correlations increased in UC in remission but decreased in active UC. The most obvious change occurred in the number of bacteria-fungi correlations, while the number of fungi-fungi correlations was insignificant among the three groups.

patients with UC, but the changes in bacterial composition were inconsistent. Some studies have reported a depletion of fecal *Firmicutes* and its members in patients with UC compared with non-IBD controls or co-habitation healthy partners[10,15,17,26,27], while others have reported opposite alterations[7]. The analysis of fecal *Bacteroids* and their members in patients with UC compared with non-IBD controls also reached different results[7,11,15,17,27]. The changes in mucosal bacteria in patients with UC are even more complicated and inconsistent than those in fecal ones[6,12,13,16]. Such confusing results may partially be attributed to the different sample sizes, ages, and races of subjects[14]; sampling biogeography; and the luminal pH values[10]. Another possible reason may be that many studies took the active and the quiescent patients with UC as a whole and analyzed them without distinction. This opinion was supported by our study as well as other studies that found different changing patterns of certain bacteria in active and quiescent UC[9,28]. Patients with UC even displayed different bacterial compositions before and after treatment, with the latter being more similar to non-IBD controls[8].

Third, the intestinal bacteria displayed a gradually decreased α-diversity and consistently changing pattern of certain bacteria when the inflammation state of active UC increased, which was represented by a gradual increase in *Proteobacteria* and *Escherichia-Shigella* and decrease in *Bacteroidetes*, *Faecalibacterium*, *Roseburia*, *Bacteroides*,

and *Eubacterium rectale group* in stool, as well as less *Methylobacterium* and *Blautia* in the mucosa. Previous studies also found that the patients with active UC with intestinal bacteria dominated by some kinds of bacteria (the members of *Proteobacteria*) exhibited more severe disease conditions than those enriched in other genera (*Blautia*, *Ruminococcus*, and other members belonging to *Firmicutes*)[17,18,29].

Despite the obvious alteration in intestinal bacteria along with the changed disease activity of UC, the bacterial composition only showed a slight or no difference when the disease type or biopsy site differed. These results agreed with most of the previous studies[11,16,19,20] except one finding that showed a significant difference between inflamed and noninflamed sites[6].

There were some commonalities among the changes of intestinal bacteria in this study. The bacteria with "protective effects" decreased while those with "deleterious effects" increased in active UC, especially in patients with moderate-severe inflammation, while in UC in remission, some of these bacteria changed in the opposite way, indicating a "correction effect" of some "protective" bacteria in recovering patients. The most representative change was the reduction of a series of genera affiliated with *Lachnospiraceae* and *Ruminococcaceae* in active UC. These are important bacteria for producing butyric acid, a substance beneficial to IBD in terms of anti-inflammation and intestinal barrier defense[30]. In addition, a decrease in the genus *Alistipes* in active UC was reported to produce bacterial sulfonolipids with potential anti-inflammatory effects[31]. Most of the genera that increased in the active UC and/or moderate-severe UC groups were opportunistic pathogens that may be harmful to gastrointestinal health. For example, *Escherichia-Shigella*[32] and *Prevotella*[33] promote inflammatory cytokine expression. In addition, *Fusobacterium*, which was more abundant in the feces of pancolitis patients, was enriched in patients with gut inflammation and was proportionally associated with colorectal cancer[34]. However, it is difficult to determine whether the change in microbiota is a cause or a consequence of UC since the bacteria depleted in patients were sometimes strictly anaerobic and failed to survive in the broken intestinal environment[30].

This study also detected fecal fungi and the intermicrobiota correlations. The diversity of fecal fungi was similar among groups, and the fungal composition only showed subtle differences at the genus level, which was in accordance with a previous study[17] but contrary to another[5]. The number of intermicrobiota correlations increased for UC in remission and decreased in active UC, with the most obvious change in the bacteria-fungi correlation, and the distribution of the correlations became uneven in patients with UC. The meaning of these results is still unclear, and more studies are needed to explore the complex changes in the intestinal fungi of IBD patients.

There were some limitations in this study. First, the sample size was insufficient, especially for the mucosa samples. Second, non-IBD controls were asymptomatic patients with colonic polyps rather than healthy people owing to ethical issues. Since the patients with colonic polyps generally had a higher onset age than patients with UC, the average ages of the controls and patients with UC were unmatched. It has been reported that age influences gut microbiota in adults, which is mainly manifested as decreases in the families of *Firmicutes* and *Bifidobacteria*, as well as an increase in *Proteobacteria* members, indicating a more proinflammatory gut microbiota phenotype in elderly people[35]. Therefore, the difference between non-IBD controls and patients with UC may be more obvious than that presented in this study. Third, many studies have implied that the gut fungal composition was significantly affected by lifestyle[36,37], but due to practical reasons, our study did not collect the information of the lifestyle in this population, which can be a confounding factor. Finally, the sequencing method in our study can only reach the genus level, and further research using the metagenomic sequencing is required to acquire more details at the species level.

CONCLUSION

Patients with active UC have a significantly different intestinal microbiota than non-IBD controls, while the difference between patients with UC in remission and non-IBD control is smaller. Disease degree of patients with active UC significantly affects the gut microbiota, and some bacteria show consistently changing patterns when the disease worsens. Some "beneficial" bacteria decrease while some "detrimental" bacteria increase in patients with active UC, especially in patients with moderate-severe inflammation. A difference was also observed in the intestinal bacteria flora collected from paired stool and mucosa samples of patients with UC. The fecal fungal

microbiota does not show evident changes compared with the controls. However, significantly different intermicrobial correlations and an unevenly distributed bacteria-fungi interaction were observed.

ARTICLE HIGHLIGHTS

Research background

The etiology of ulcerative colitis (UC) is still unclear, and dysbacteriosis may be a crucial environmental factor. Numerous studies have focused on the variations in intestinal microbiota diversity and composition of patients with UC.

Research motivation

Existing studies concerning UC and intestinal microbiota have arrived at various conclusions. A further understanding of the constitution and changes of the intestinal microbiota in patients with UC is necessary to elucidate the possible pathogenesis and investigate potential treatments.

Research objectives

To analyze the relationship between different clinical features and the intestinal microbiota, including bacteria and fungi, in Chinese patients with UC.

Research methods

Gene amplicon sequencing of 16S rRNA gene and fungal internal transcribed spacer gene was used to detect the intestinal microbiota composition. Alpha diversity, principal component analysis, similarities analysis, and Metastats analysis were employed to evaluate differences among groups.

Research results

A total of 89 patients with UC and 33 controls were enrolled. A significant difference existed between the fecal and mucosal bacteria, and the α-diversity of intestinal bacteria and the relative abundance of some families decreased with the increasing severity of bowel inflammation. More intermicrobial correlations in UC in remission than in active patients were observed, and the bacteria-fungi correlations became single and uneven in patients with UC.

Research conclusions

Patients with active UC have a significantly different intestinal microbiota in terms of various sample types and disease activities. Significantly different intermicrobial correlations and an unevenly distributed bacteria-fungi interaction were observed.

Research perspectives

There is still a need to collect subjects to expand the sample size and further include data from other parts of China. And metagenomic sequencing method may be needed for more details at the species level in future research.

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CASE REPORT

HER2-positive adenocarcinoma arising from heterotopic pancreas tissue in the duodenum: A case report

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Author contributions: Hirokawa YS contributed to pathological diagnosis and manuscript drafting; Tanaka K and Sakurai H performed surgery; Iwata T and Okugawa Y contributed to medical treatment; Watanabe M was responsible for the manuscript revision; all authors made final approval for the manuscript to be submitted.

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Abstract

BACKGROUND

Adenocarcinoma originating from heterotopic pancreas tissue is a rare disease. Furthermore, to our knowledge, no HER2-positive cases in the duodenum have been reported in the scientific literature nor has the efficacy of trastuzumab treatment for the disease been reported.

CASE SUMMARY

A 65-year-old woman whose clinical diagnosis was unresectable advanced duodenal cancer with HER2 overexpression responded well to trastuzumab chemotherapy. The main tumor in the duodenum reduced drastically. The patient underwent pancreaticoduodenectomy and lymph node dissection. A small number of cancer cells remained in the submucosal layer of the duodenum and pancreas head. After histological and immunohistochemical examination, the patient was diagnosed with duodenal adenocarcinoma originating from heterotopic pancreas tissue.

CONCLUSION

Trastuzumab treatment is effective in HER2-positive adenocarcinoma originating from heterotopic pancreas tissue in the duodenum.

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Core Tip: Adenocarcinoma that develops from heterotopic pancreas tissue in the duodenum is a very rare disease. Therefore, standard chemotherapy regimens to treat these patients have not been established. A 65-year-old woman presented with unresectable duodenal cancer with HER2 overexpression. Because the patient responded well to trastuzumab chemotherapy, pancreatoduodenectomy was performed. Histology and immunohistochemistry assessments revealed that the cancer developed from heterotopic pancreas tissue in the duodenum. The findings of this case suggest that HER2 status should be examined in duodenal adenocarcinoma to determine the indication for targeted therapy. Trastuzumab chemotherapy is effective for heterotopic pancreatic adenocarcinoma but not orthotopic pancreatic cancer.

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INTRODUCTION

Heterotopic pancreas is a relatively rare event detected in 0.25% of gastrointestinal tract surgeries and in 0.6 to 13.7% of autopsy cases[1,2]. Malignant transformation was observed only in 0.7% to 1.8% of heterotopic pancreas cases[3,4]. In the literature, 16 reports of malignant tumors arising from duodenal heterotopic pancreas tissue were confirmed[5-7]. Some patients received chemotherapy, but information about the efficacy of the chemotherapy applied is limited. We describe the first case of HER2-positive adenocarcinoma originating from heterotopic pancreas tissue in the duodenum and the therapeutic effect of trastuzumab treatment in this patient.

CASE PRESENTATION

Chief complaints

The patient presented with epigastric discomfort that had persisted for 3 mo.

History of present illness

A 65-year-old woman complained of abdominal discomfort and had visited the hospital previously. Esophagogastroduodenoscopy revealed an advanced type 4 lesion in the pylorus of the stomach. The patient received a detailed medical examination and treatment at the current hospital.

History of past illness

The patient had an appendectomy due to acute appendicitis at the age of 15.

Personal and family history

The patient had no smoking or alcohol intake history. There was no family history of cancer.

Physical examination

The abdomen was soft and without tenderness.

Laboratory examinations

Initial laboratory data revealed a hemoglobin level of 7.2 g/dL, a white blood cell

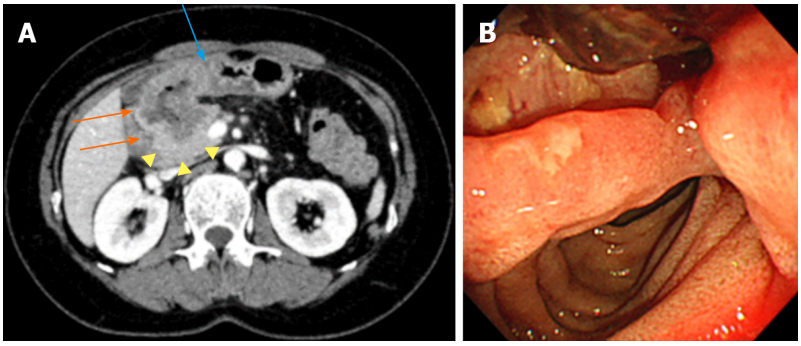


Figure 1 Abdominal computed tomography and gastroduodenoscopy. Images were obtained before the start of treatment. A: An axial image shows an irregular circumferential mass in the first portion of duodenum (orange arrows), with transmural extension to pylorus (blue arrow) and direct extension to pancreas head (yellow arrow heads); B: During gastroduodenoscopy, mass lesion with ulcer was visible in the first portion of duodenum.

count of 5700 cell/μL, and a platelet count of 3.95 × 10^5/μl. The creatinine level was 0.64 mg/dL, total bilirubin level was 0.44 mg/dL, aspartate aminotransferase level was 11 U/L, alanine aminotransferase level was 9 U/L, albumin level was 3.6 g/dL, and amylase level was 75 U/L (normal range 37 to 125 U/L). The level of the carcinoembryonic antigen tumor marker was 445.4 ng/mL, the cancer antigen 125 level was 317.3 U/mL, the squamous cell carcinoma antigen level was 4.1 ng/mL, and the carbohydrate antigen 19-9 level was less than 2 U/mL.

Imaging examinations

An abdominal computed tomography (CT) scan demonstrated wall thickening in the duodenal first portion extending toward the pylorus and pancreatic head (Figure 1A). Lymph node swelling in the common hepatic artery and in the portal vein and venous tumor thrombosis in the pancreatic head were also observed. There was no gross tumor mass in the orthotopic pancreas. The patient was referred for gastroduodenoscopy. The tumor was located in the first portion of the duodenum with ulcer formation (Figure 1B). The stomach pylorus was stenotic due to the extrinsic compression of the tumor. Histologic evaluation of the duodenum biopsy specimens revealed tubular adenocarcinoma, and HER2 overexpression was detected by immunohistochemistry (Figure 2). The clinical diagnosis of unresectable duodenal cancer was made based on cancer invasion of the pancreas and portal vein tumor thrombosis; thus, stomach bypass surgery and gastrojejunostomy were performed. Capecitabine and cisplatin plus trastuzumab chemotherapy was applied due to the potential efficacy of this treatment for HER2-overexpressing gastric cancer cells reported in the Trastuzumab for Gastric Cancer (ToGA) study. After 1 cycle of treatment, the patient developed renal impairment due to the side effects of cisplatin. The drug combination was changed to capecitabine and oxaliplatin plus trastuzumab. To assess the effects of treatment, a CT scan was performed after 2 cycles of capecitabine and oxaliplatin plus trastuzumab administration, and tumor size and lymph node swelling were dramatically reduced (Figure 3). The same chemotherapy was continued for two more cycles, and then pancreatoduodenectomy was conducted. No obvious tumor was visible in the duodenum or liver and there was no peritoneal metastasis.

Pathological examination

The histological evaluation of the biopsy specimens from the gastroduodenoscopy revealed an adenocarcinoma mass predominantly located in the duodenal submucosa (Figure 2). The cancer cells were strongly positive for HER2 membranous expression on immunohistochemical analysis (+3). On gross examination of the resected duodenum, no discrete mass lesion was visible, but a flat, slightly elevated mucosal fold was present in the first portion of the duodenum. On histological assessment, carcinoma cells were localized in the duodenal submucosal layer, forming small nodules, and in the pancreas head and pancreaticoduodenal lymph nodes (Figure 4A and B, data not shown). Heterotopic pancreas tissue was located close to the carcinoma cells within the submucosal layer and laminar muscularis mucosa of the duodenum, composed of acinar and duct structures (Figure 4C). In the immunohistochemical analysis, the cancer cells showed positive staining for cytokeratin (CK)7, CK20, MUC1 and MUC5AC and negative staining for MUC2 and CDX2 (Figure 5A-D and data not shown). Positive staining for MUC6, a specific marker for Brunner gland adenocar-

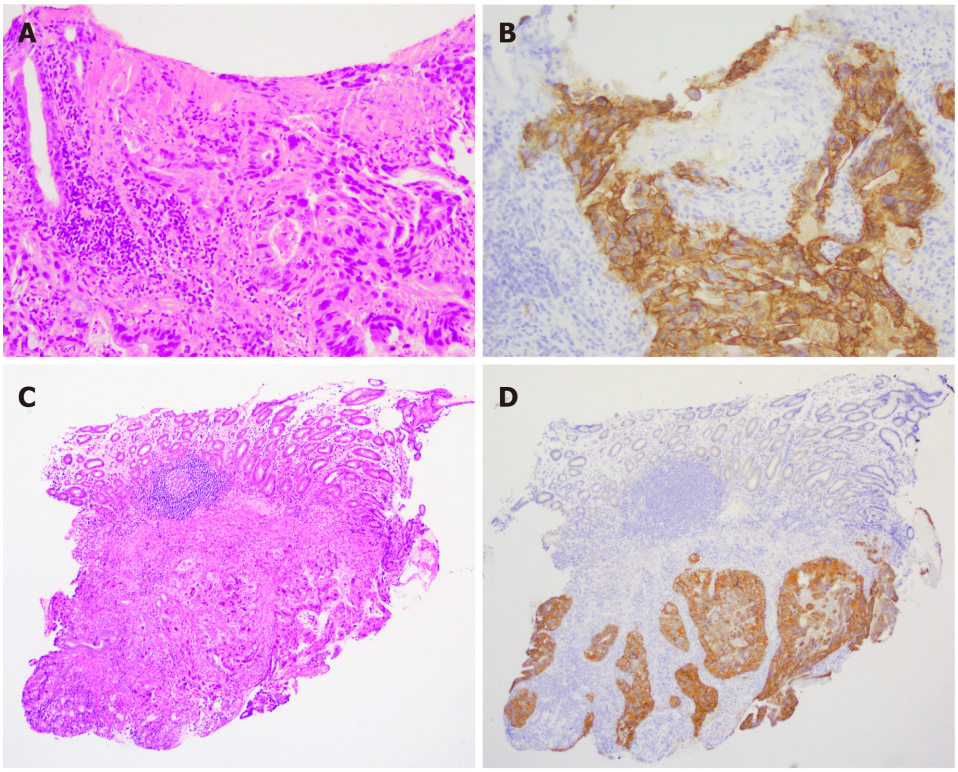


Figure 2 Histological findings of biopsy specimens from duodenal lesion. A: Adenocarcinoma cells proliferate in the proper mucosal and submucosal layer with ulcer formation (HE staining, × 200); B: Adenocarcinoma cells show strong HER2 membranous expression (× 200); C and D: Adenocarcinoma cells proliferate in the submucosal layer with strong HER2 protein expression (× 54).

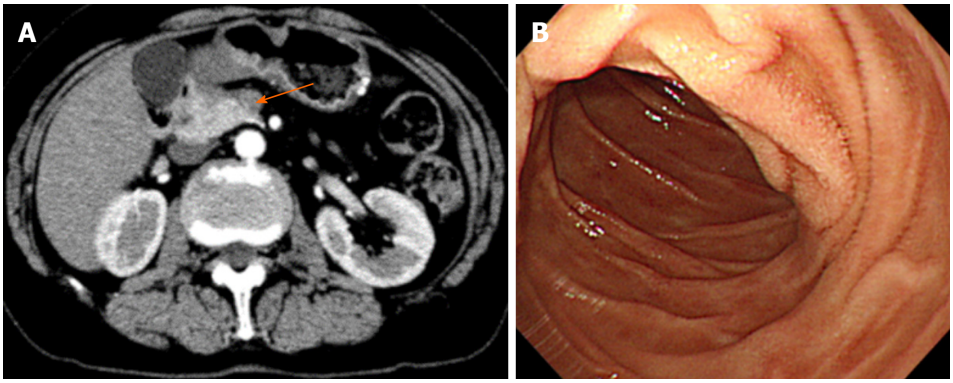


Figure 3 Abdominal computed tomography and gastroduodenoscopy. Images were obtained after 2 cycles of trastuzumab treatment. A: An axial image shows an irregular mass lesion in the pancreas head (orange arrows), duodenal and pylorus lesion are disappeared; B: During gastroduodenoscopy, mass and ulcer lesion were vanished in the first portion of duodenum.

cinoma, was positive in the Brunner glands but negative in the cancer cells (Figure 5E) [8].

FINAL DIAGNOSIS

These findings indicated that the carcinoma cells were of pancreatic duct origin[9-11] and are consistent with the diagnosis of adenocarcinoma arising from heterotopic pancreas tissue within the duodenum. The residual cancer cells were positive for HER2 on immunohistochemical analysis (Figure 5A).

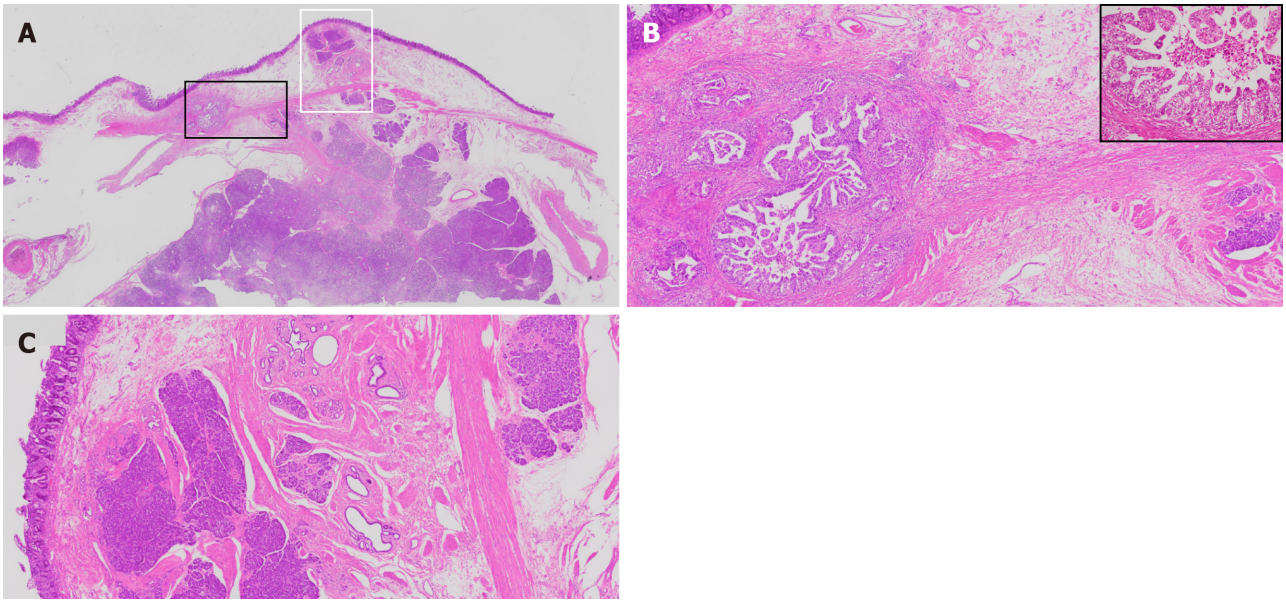


Figure 4 Histological findings of resected duodenum. A: Heterotopic pancreas tissues are located in the submucosal layer and the laminar muscularis mucosa (HE staining, x 0.08). Adenocarcinoma cells are positioned in closely to the heterotopic pancreas tissue (× 0.08); B: On magnified image of the black square in Panel A, papillary like growth of the adenocarcinoma is present in vicinity of the heterotopic pancreas tissue (HE staining, × 10 and inset × 200); C: On magnified image of the white square in Panel A, submucosal heterotopic pancreas tissue is composed of acinar and duct structure (HE staining, × 10).

TREATMENT

Various chemotherapies were given after surgery as follows: 1 cycle of capecitabine and oxaliplatin and 6 cycles of capecitabine. In the CT examination for evaluating chemotherapy, retroperitoneal lymph node enlargement, implying cancer metastasis, was revealed. Progressively, 3 cycles of paclitaxel, 4 cycles of S-1 and oxaliplatin plus trastuzumab, and 2 cycles of S-1 plus trastuzumab were administered. Thereafter, trastuzumab monoadministration was conducted. Chemotherapy-induced side effects guided the changes in drug selection.

OUTCOME AND FOLLOW-UP

Twenty-four months after the operation, the patient presented with enlarged retroperitoneal lymph nodes and was diagnosed with progressive disease according to the Evaluation Criteria in Solid Tumors. Due to the slow progression of the retroperitoneal lymph nodes, trastuzumab monoadministration was continued. When the retroperitoneal lymph nodes kept increase in size, nivolumab administration would be considered according to the gastric cancer chemotherapy regimens.

DISCUSSION

We describe a case of HER2-positive adenocarcinoma that developed in heterotopic pancreas tissue in the duodenum and outline the efficacy of HER2-targeting therapy for this disease for the first time in the English literature. Guillou *et al*[3] proposed the following three conditions for the definition of carcinoma arising from heterotopic pancreas tissue: (1) The tumor must be found within or close to the ectopic pancreatic tissue; (2) Direct transition between pancreatic structures and carcinoma must be observed; and (3) The nonneoplastic pancreatic tissue must comprise fully developed acini and ductal structures. In our case, the second condition, the transition between pancreatic structures and carcinoma, was not fulfilled completely; however, the desmoplasia of pancreatic cancer destroys the tissue architecture, making it impossible to identify the histological transition. Guillou *et al*[3]'s report included inconsistent statements; the term "close to" was used in the first condition and "direct transition" was used in the second. Furthermore, given the expanding knowledge within the field of pancreatic cancer immunohistochemistry since Gulliou *et al*[3]'s study was

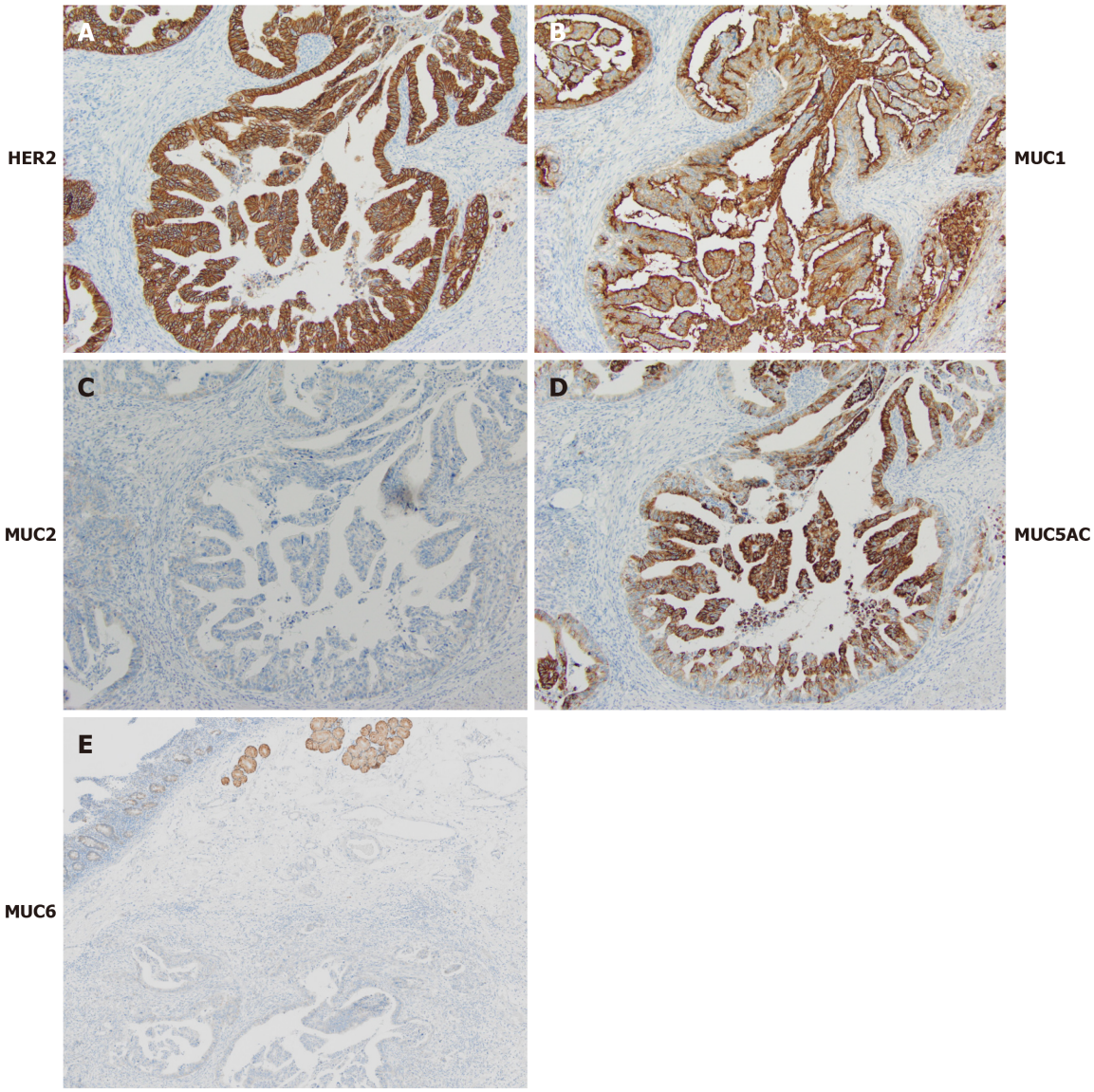


Figure 5 Immunohistochemical staining in adenocarcinoma cells in the submucosal layer. A: Strong HER2 expression is localized in the cell membrane (× 100); B-E: Adenocarcinoma cells express MUC1 and MUC5AC but no MUC2 or MUC6 protein (B-D: × 100; E: × 3.5).

published, we propose a replacement for the second Gulliou *et al*[3] criterion as follows: Direct transition between pancreatic structures and carcinoma is observed; alternatively, cancer cells express proteins indicating pancreatic origin.

Since chemotherapy against HER2-positive cancer improved the outcome of breast cancer patients, an extensive search was conducted to elucidate the incidence of HER2-expressing pancreatic cancer (ductal adenocarcinoma). The incidence of pancreatic cancer with +3 membranous HER2 protein expression ranges from 6% to 12% in the literature[12-14]. Using the same approach to investigate the use of Herceptin combined chemotherapy for metastatic pancreas cancer, Safran *et al*[13] reported modest a benefit to overall survival, but Harder *et al*[14] found no such beneficial results. Harder *et al*[14] conducted a phase II trial of trastuzumab and capecitabine, the same drug combination administered to our patient, for metastatic pancreatic cancer. The therapeutic efficacy of HER2 chemotherapy in the current case supports the theory that HER2 overexpression drives cancer progression. The mechanism of cancer cell proliferation might differ between heterotopic and orthotopic pancreatic cancer. Given that HER2 is one of the EGFR family receptors, HER2 shares the same two main downstream signaling pathways as other EGFR receptors, the RAS-MAPK and PI3K-AKT-mTOR pathways[15]. The most reliable explanation of the failure of HER2-targeted therapy is that many pancreatic cancers possess KRAS mutations, which lead to cancer cell proliferation regardless of HER2 inactivation. The cancer in the current case had no KRAS mutation, which supports the rationale for HER2-targeted chemotherapy (data not shown). However, the current case also revealed the

limitation of the therapy, since residual cancer was observed in the submucosal layer of the duodenum, lymph nodes and desmoplastic stroma in the pancreatic head. Similar to orthotopic pancreatic ductal adenocarcinoma, in the current case, the cancer cells penetrated the pancreatic parenchyma and developed a tumor microenvironment that supported cancer cell growth, invasion, and metastasis, which contribute to therapeutic resistance[16].

Trastuzumab therapy is the first-line treatment for patients with HER2-overexpressing cancer, but the mechanism of therapy resistance has not been completely established. Biological heterogeneity or clonal evolution could be the underlying mechanism. By analyzing the mechanism involved in trastuzumab resistance, targeted capture sequencing of the circulating tumor DNA (ctDNA) of advanced gastric cancer patients and a clone structure reconstruction approach were conducted[17]. Most of the gene mutation clones were concentrated in the MAPK and RAS pathways. This approach to identifying cancer gene mutations with ctDNA analysis could help to identify new targeted therapies for trastuzumab-resistant cancer patients.

CONCLUSION

HER2-positive unresectable adenocarcinoma that developed from heterotopic pancreas tissue in the duodenum was treated with trastuzumab. HER2-targeted treatment was effective for reducing the tumor volume sufficiently for surgical resection.

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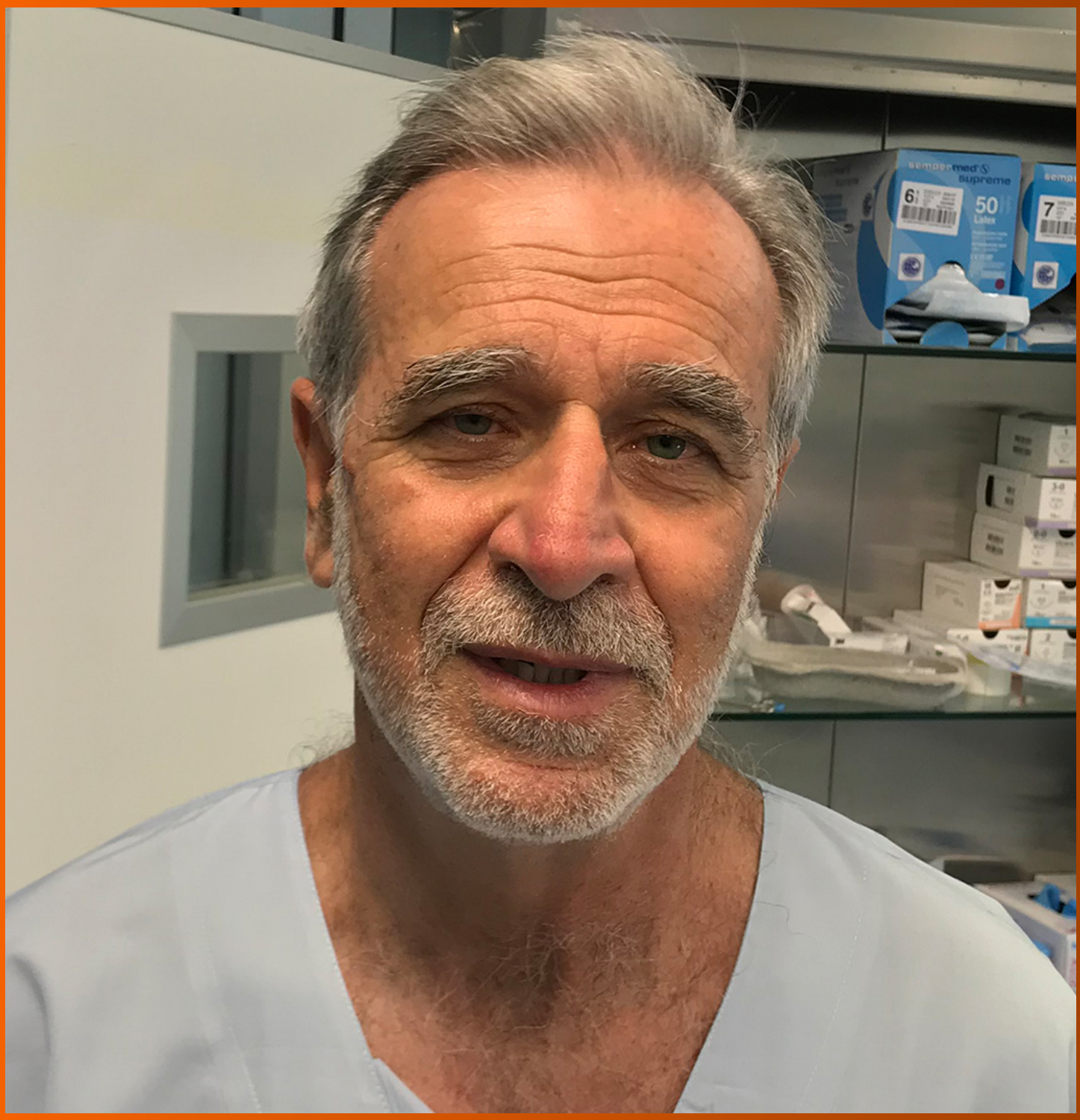
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REVIEW

Ischemic bowel disease in 2021

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Abstract

Ischemic bowel disease (ISBODI) includes colon ischemia, acute mesenteric ischemia (AMI) and chronic mesenteric ischemia (CMI). Epidemiologically, colon ischemia is the most common type followed by AMI and CMI. There are various risk factors for the development of ISBODI. Abdominal pain is the common presenting symptom of each type. High clinical suspicion is essential in ordering appropriate tests. Imaging studies and colonoscopy with biopsy are the main diagnostic tests. Treatment varies from conservative measures to surgical resection and revascularization. Involvement of multidisciplinary team is essential in managing ISBODI. Although open surgery with revascularization plays an important role, recently there is an increasing interest in percutaneous endovascular treatment.

Key Words: Colon ischemia; Ischemic colitis; Acute mesenteric ischemia; Chronic mesenteric ischemia; Endovascular treatment of acute and chronic mesenteric ischemia

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Core Tip: Ischemic bowel disease (ISBODI) includes a spectrum of diseases due to inadequate blood supply to the bowel wall. In this review, we will be discussing the epidemiology, risk factors, pathophysiology, clinical aspects, investigations, current management protocols and prognosis of each type of ISBODI.

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INTRODUCTION

Ischemic bowel disease (ISBODI) is the most common vascular disorder of the gastrointestinal tract. ISBODI results from inadequate supply of oxygenated blood to any part of the bowel wall. ISBODI is a heterogenous group of disorders that can be divided into small intestinal ischemia or mesenteric ischemia, and large intestinal ischemia or colonic ischemia (CI) or ischemic colitis (IC). Clinically mesenteric ischemia is further classified as acute mesenteric ischemia (AMI) and chronic mesenteric ischemia (CMI). Although ISBODI is not that common in clinical practice, the morbidity and mortality can be high if this entity is not recognized and treated emergently.

ISBODI IN 2021

Epidemiology

CI is the most common entity of ISBODI. The exact epidemiology of ISBODI is not known. One study showed that the overall age-adjusted and sex-adjusted incidence of CI was about 6.1 cases *per* 100000 person-years in 1976-1980 and it increased by about 4-fold to 22.9/100000 person-years over the course of few decades (2005-2009)[1]. The prevalence of AMI is 1 in 1000 hospital admissions, whereas the prevalence of CMI is 1 in 100000 individuals[2]. The incidence of CMI is probably only 2-3 *per* 100000 persons *per* year[3]. AMI represents an uncommon cause of acute abdominal pain as the overall incidence of AMI is 0.09% to 0.2% of all acute surgical admissions[4].

Risk factors

ISBODI is commonly seen in the elderly population with mesenteric vascular atherosclerosis[5]. Other risk factors include diabetes mellitus, hypertension, coronary artery disease, peripheral artery disease, atrial fibrillation, congestive heart failure, recent myocardial infarction, shock, chronic renal failure requiring hemodialysis, severe dehydration, chronic obstructive lung disease, irritable bowel syndrome, sickle cell crisis with microvascular occlusion, rheumatic autoimmune diseases, substance abuse like amphetamine abuse or cocaine abuse, long distance running, hereditary and acquired thrombophilia[6]. Iatrogenic intestinal ischemia can occur post-surgically (abdominal aortic aneurysm surgery, colectomy, bypass surgery), due to dislodgement of intravascular devices, and secondary to use of certain medications–estrogen, digoxin, danazol, alosetron, pseudo ephedrine, vasopressin, Gold, psychotropic drugs, sumatriptan, serotoninergic agonists and anatagonists immunomodulators, laxatives and non-steroidal anti-inflammatory drugs[7,8]. Enterocolonic ischemia may also be due to systemic or infectious vasculitis, fibromuscular dysplasia, radiation and amyloidosis[9]. Colonic obstruction due to neoplasm, diverticular disease, adhesion, volvulus, intussusception, internal hernia with obstruction or fecal impaction may also contribute to CI although in most cases the precipitating cause remains unidentified. The CI usually occurs proximal to the obstruction[10].

There are several physiological factors which can make the colon more susceptible to ischemia. There is less blood flow to the colon (*per* 100 g of tissue) as compared to other parts of the gastrointestinal tract. Functional colonic motor activity further decreases colonic blood flow. The colon also depends on collateral circulation frequently. During hypotensive episodes, the colon cannot autoregulate well compared with the rest of the gastrointestinal tract[11].

Pathophysiology

It is important to have a clear knowledge of the vascular supply and venous drainage of the intestine to understand the pathophysiology of ISBODI. Small intestine and large intestine derive their blood supply mainly from the superior mesenteric artery (SMA) and the inferior mesenteric artery (IMA). Proximal part of the duodenum (up to the level of ampulla of Vater) receives its blood supply from the celiac artery (CA) *via* gastroduodenal artery (GDA) (a branch of hepatic artery) and superior pancreaticoduodenal artery (a branch of GDA)[12]. Rest of the small bowel and right two-third of the colon (up to splenic flexure) are supplied by SMA. Numerous branches arise from the SMA to supply the jeunum and ileum. These branches pass between the layers of the mesentery and form anastomotic arcades from which straight arteries called vasa recta arise to supply jejunal and ileal walls. Jejunal arterial arcades are less in number but they have long vasa recta. Ileal arterial arcades are more in number but they have short vasa recta (Figures 1 and 2)[13]. As a result, jejunum is more

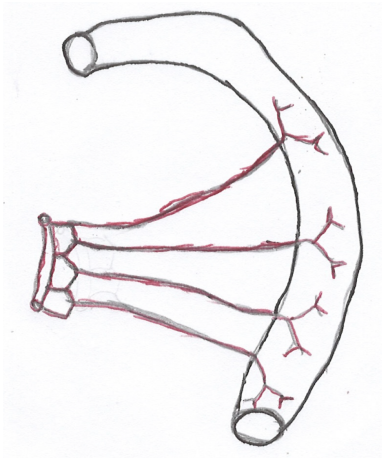


Figure 1 Jejunal arcades and long vasa recta.

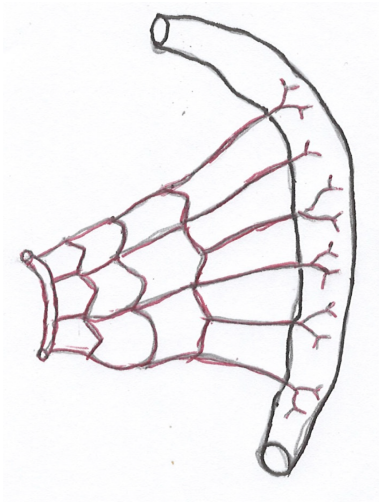


Figure 2 Ileal arcades and short vasa recta.

vulnerable to ischemic injury than ileum.

Left one-third of the colon and upper two-third of the rectum are supplied by IMA (most diminutive of 3 mesenteric arteries). Lower one third of the rectum is supplied by middle rectal artery (a branch of anterior division of internal iliac artery) and inferior rectal artery (a branch of internal pudendal artery). The transverse colon is mostly supplied by the middle colic artery which is a branch of the SMA. It may also receive blood supply from the marginal artery which is the anastomotic arcade between the left colic and right colic arteries. There are 3 watershed zones in the colon that receives blood supply from the distal branches of two large arteries. As they have the fewest vascular collaterals, they are prone to develop non-occlusive IC at the watershed zones when blood flow decreases. The watershed zones include the splenic flexure or Griffith's point, rectosigmoid region or Sudeck's point and the ileocecal region[14]. The small bowel mucosa is more sensitive to ischemia as compared to colonic mucosa but it has tremendous regenerative potential. On the other hand, colonic mucosa is less easily damaged by ischemia but once destroyed, slow recovery occurs[15].

Of 25% of resting cardiac output and 35% of post-prandial cardiac output go to the mesenteric circulation[16]. Mucosa and submucosa of the intestine receive 70% of mesenteric blood while muscularis propria and serosa receive the remainder (30%) of mesenteric blood. Mesenteric ischemia does not occur until the blood supply is reduced by more than 50%[17] or patient's mean arterial pressure does not go below 45 mmHg[18]. Significant injury to the intestine may not occur even if a 75% reduction in blood flow occurs for up to 12 h but complete occlusion of blood flow will cause bowel wall infarction within 6 h.

The venous drainage follows the arterial supply forming venae rectae and venous arcades so that duodenal venous drainage occurs *via* the splenic vein or superior mesenteric vein (SMV); jejunal, ileal and proximal (two-third) colonic venous drainage occurs *via* SMV, and distal (one-third) colonic venous drainage occurs *via* inferior mesenteric vein. Ultimately, these veins drain into the portal vein[19]. Atherosclerosis, thromboembolism, hypoperfusion (as a result of arrhythmia, heart failure, shock), hypercoagulable state, vasculitis, venous occlusion or any mechanical obstruction to the mesenteric blood vessels may to lead to ISBODI[20]. In most cases of SMA embolism, duodenum, proximal jejunum and transverse colon get spared as emboli lodge 6 to 8 cm beyond its origin, often beyond the origin of middle colic artery. Emboli lodge into the SMA more easily possibly because of its large diameter and its origin from the aorta at an acute angle[21]. On the other hand, SMA thrombosis generally occurs at the origin of SMA at the site of severe atherosclerotic narrowing [22]. Proximal colon may become ischemic because of involvement of ileocolic artery. Mesenteric venous thrombosis (MVT) generally involves the SMV but inferior mesenteric veinous (IMV) thrombosis can occur in up to 11% of all cases of MVT[23]. Venous thrombosis leads to intestinal edema and increased vascular resistance leading to bowel ischemia. Non-occlusive mesenteric ischemia (NOMI) results from mesenteric vasoconstriction associated systemic hypotension and low flow state in the splanchnic circulation. NOMI can cause small bowel as well as proximal CI and necrosis because of involvement of ileocolic artery.

AMI occurs due to sudden decrease in blood flow to varying portion of the small intestine and/or colon. The ischemic process can be severe enough to cause instant bowel wall infarction. The main mechanism of ischemic injury is hypoxia and cellular acidosis due to local accumulation of lactic acid and other cellular metabolic by-products secondary to anerobic glycolysis. Complete ischemia may result in infarction of the bowel wall supplied by the blood vessel whereas relative ischemia generally causes cellular dysfunction[24]. If the vascular obstruction is relieved soon after ischemic injury, reversibly injured cells may recover but if reperfusion occurs late, irreversibly damaged cells may not recover and this may lead to inflammation and bleeding due to release of reactive oxygen species and endothelial damage that occurred during the ischemic period. The restored blood flow to the ischemic area can cause further tissue damage called reperfusion injury as the white blood cells (WBC) in the blood release free radicals and interleukins; oxygen delivery to the ischemic tissue damages plasma membrane, DNA and cellular protein; and plasma membrane injury may release more free radicals. WBC can cause further ischemia if they bind to the endothelial cells of capillaries[25]. The pathophysiological process correlates well with the clinical scenario. In the first phase, patient feels abdominal pain because of bowel ischemia and hyperperistalsis. In the second phase (after 3 to 6 h), patient feels better as the intestine becomes aperistaltic and the intramural pain receptors become damaged by ischemia. In the third phase, mucosal necrosis occurs followed by infarction of the bowel wall. Bacterial translocation leads to gangrene of the intestine, diffuse peritonitis, sepsis and multi-organ failure[26].

CI can cause reversible damage or irreversible damage[27]. Reversible damage occurs in 50% of cases and this includes: (1) Reversible ischemic colopathy due to submucosal or intramural hemorrhage in two-third of cases; and (2) Transient segmental IC in one-third of cases. Irreversible damage may cause chronic ulcerative IC in 20%-25% of cases, ischemic colonic stricture in 10%-15% of cases, colonic gangrene in about 15% of cases, and fulminant universal colitis or pancolitis in only 1% of cases[28]. Old age, long duration of subjective symptoms and prolonged Laboratory (Lab) abnormalities (elevated white blood cell count or erythrocyte sedimentation rate) may predict the development of chronic ulcerative IC or ischemic colonic stricture. 85% of cases of CI are non-gangrenous, transient and self-limiting [29]. In 95% of cases, CI occurs due to sudden but transient reduction in blood flow to the colon due to non-occlusive causes and as a result, the watershed zones are affected [30]. Most of the time, the cause of non-occlusive ischemia remains unidentified and it is thought to be due to localized non-occlusive ischemia secondary to small-vessel disease (Type I disease). Rarely, episodes of non-occlusive ischemia secondary to systemic hypotension are identified (Type II disease). Occlusive ischemia due to atheromatous emboli or vasculitis affects short segment of the colon. CI can affect any part of the colon but the left colon is involved in two-third of cases[31]. Segmental involvement of the colon is commonly observed, particularly the splenic flexure, the descending colon and the sigmoid colon are the commonest sites of involvement. Isolated right colon ischemia can occur in about 10% of cases. Right colonic involvement generally occurs in patients with chronic renal failure requiring hemodialysis and is associated with severe IC[32].

Ischemic injury first occurs in the colon mucosa and it is most commonly seen on the antimesenteric side. Subsequently the ischemic changes may extend to the outer layers of the colon wall.

CMI occurs as a result of gradual decrease in blood flow to the intestine. As a result, numerous collaterals develop in the mesenteric circulation[33]. CMI is almost always associated with athererosclerosis. Although the prevalence of asymptomatic chronic stenotic mesenteric disease varied from 3% (below the age of 65 years) to 18% (above the age of 65 years) on doppler sonography in some studies[34], the incidence of CMI is low as mentioned before. Asymptomatic single vessel disease is more common in CA (81%) than in the SMA (19%). Generally, two out of the three major blood vessels (CA, SMA and IMA) need to be stenotic to produce ischemic symptoms. But patients may develop symptoms even with single vessel involvement[35] and many patients with two vessel disease may remain asymptomatic for many years because of extensive collaterals in the mesenteric circulation[36]. The most common cause of single vessel involvement is CA compression syndrome or median arcuate ligament syndrome where eccentric compression of the CA and/or celiac ganglion occurs by the median arcuate ligament and diaphragmatic crura[37]. The pancreaticoduodenal artery arcades, GDA, dorsal pancreatic artery and occasionally the arc of Buhler (embryonic anastomotic branch between 10th and 13th ventral segmental arteries) are the most common collaterals between the CA and the SMA[38]. On the other hand, the marginal artery of Drummond (anastomosis between the terminal branches SMA and IMA forming a continuous arterial arcade along the inner border of the colon) and arc of Riolan/meandering mesenteric artery (arterioarterial anastomosis between the middle colic branch of SMA and left colic branch of IMA running close to the root of the mesentery) are the most common collaterals between the SMA and IMA[39]. The exact association of severity of mesenteric arterial stenosis with collaterals formation and symptom development is not known.

Chronic NOMI is another entity found in 13% to 16% of all patients with CMI[40]. Its clinical presentation is similar to chronic occlusive mesenteric ischemia but vascular occlusion is not identified. The exact etiology is not known but possible pathophysiological mechanisms include vasospasm, micro-embolisms, shunting, cardiac or pulmonary insufficiency and autonomic dysfunction.

Clinical aspects

AMI is a life threatening potentially fatal abdominal and vascular emergency due to diminished blood flow in the superior mesenteric blood vessels. Patients are generally above the age of 60 and women are 3 times more commonly affected as compared to men. Although the clinical presentation is similar, the etiology could be occlusive or non-occlusive ischemia. SMA embolism is the most common cause (40%-50%) followed by SMA thrombosis (15%-25%) followed by MVT (5%-15%)[41]. NOMI occurs in about 5 to 15% of cases AMI[42].

Patients generally present with sudden onset of severe sharp abdominal pain that is generally accompanied by nausea and vomiting. The pain is out proportion to physical signs. In the early phase, abdomen is generally soft and non-tender but auscultation may reveal hyperactive bowel sound. Tenderness to palpation occurs when the entire bowel wall becomes ischemic. In late phase (within 12 h of onset) rebound tenderness (Blumberg's sign), rigidity and guarding indicate bowel wall infarction. At this stage, bowel sounds become hypoactive or absent, patient develops bloody diarrhea, fever and shock.

High degree of clinical suspicion is necessary to establish the diagnosis of AMI. A focused history is important to evaluate the underlying pathophysiology of AMI. Patients with atrial fibrillation, valvular heart disease, congestive heart failure, recent myocardial infarction and bacterial endocarditis are at increased risk of mesenteric embolism. About 50% of patients with embolic AMI have atrial fibrillation. Prior history of embolism is present in about one third of patients of embolic AMI[43]. The clinical triad of (1) Severe abdominal pain out of proportion to physical signs; (2) Spontaneous bowel movement (vomiting and/or non-bloody or bloody diarrhea); and (3) Obvious source of emboli such as atrial fibrillation or recent myocardial infarction may be present in 40% to 80% cases of SMA embolism[44]. History of chronic abdominal pain in the postprandial period, progressive weight loss and vascular procedures in the mesenteric arteries may be present in patients with mesenteric arterial thrombosis. Chronic and progressive symptoms appear in mesenteric arterial thrombosis is because of preexisting atherosclerosis plaque affecting mesenteric artery is the most common finding. NOMI should be considered in patients with hypotension and generalized (diffuse) abdominal pain. As NOMI generally occurs in critically ill patients on ventilator, the presentation can be silent. Gastrointestinal bleeding or

unexplained abdominal distention can be the only presentation of NOMI. MVT is suspected when patients with thrombophilia (hereditary or acquired) or coagulopathy present with abdominal pain (84% of cases), diarrhea (42% of cases), nausea/vomiting (33% of cases) or gastrointestinal bleeding (10% of cases)[45]. The author finds these clinical suspicions very useful in categorizing the pathophysiologic form of AMI that can guide the investigation and therapy.

CI is mostly a benign and self-limiting disease in most of the time although a minority of cases can be severe. Most of the patients with non-iatrogenic causes are older than 60 years old with a slight female predilection. Rarely, IC can also occur in young individuals due to substance abuse, vasculitis, long distance running, thrombophilia, sickle cell crisis with microvascular occlusion, irritable bowel syndrome and iatrogenic causes as mentioned before. Chronic constipation and prior history of abdominal surgery are common in both young and old patients with CI[46]. One study found that 6.8% of patients had a recurrence of CI with a mean follow-up of 4.5 years [47]. The recurrence is more common in patients with hereditary thrombophilia (deficiencies of protein C, protein S, antithrombin III, and factor V Leiden mutation), anti-phospholipid syndrome, active smoking, abdominal aortic aneurysm, coronary artery disease and elevated serum creatinine[48,49].

Patients with CI generally present with sudden onset of mild to moderate cramping pain over left lower quadrant of the abdomen or hypogastrium, and urgency to defecate followed by hematochezia within 24 h. The bleeding is not severe enough to require blood transfusion. Patients with right colon ischemia usually present with hypogastric pain rather than hematochezia[50]. Physical examination may reveal mild to moderate tenderness over the left lower quadrant or over the involved segment. The usual course of the disease is benign in non-gangrenous and non-fulminant CI. Patients feel better with resolution of abdominal pain and hematochezia in few days time. Persistent symptoms more than few days may indicate development of chronic CI *i.e.* chronic ulcerative IC or ischemic colonic stricture. Gangrenous colitis or fulminant pancolitis is generally recognized by marked abdominal tenderness, rebound tenderness, hypotension or shock. Absence of hematochezia and development of hypoalbuminemia may predict the development of gangrenous changes of CI[51].

CMI also called abdominal angina or intestinal angina or migraine abdominale is mostly seen in patients older than 60 years of old and is 3 times more common in females than in males[52]. Patients generally present with the triad of sharp post-prandial epigastric or mid-abdominal pain, sitophobia (fear of eating food) and chronic weight loss[53]. Patient starts feeling abdominal pain 15 to 30 min after a meal and the pain lasts for about half an hour to 2 h[54]. As the diseases progress, abdominal pain becomes dull-aching and chronic. Patients may also develop nausea, vomiting, diarrhea or constipation[55]. Physical examination may reveal abdominal bruit. But only 16% to 22% of patients with CMI have the classic triad of post-prandial abdominal pain, weight loss and abdominal bruit[56].

Investigations

AMI–At the present time, there is no specific Lab test which can identify AMI. But evidence of hemoconcentration, elevated white cell count, high anion gap lactic acidosis and positive D dimer test (fibrinolytic marker) in the presence severe abdominal pain disproportionate to physical sign may indicate AMI. Leucocytosis is the most common Lab finding seen in > 90% patients with AMI followed by lactic acidosis seen in 88% of cases of AMI[57]. But as the liver can metabolize large amount of L-lactic acid from the porto-mesenteric circulation and lactic acidosis can occur in dehydrated patients, it cannot reliably differentiate intestinal ischemia from infarction in the absence of appropriate clinical scenario. D-dimer can be considered as an exclusion test as in one study no patient with normal D-dimer had AMI[58]. Hyperamylasemia, hyperkalemia and hyperphosphatemia can occur when mesenteric ischemia progresses to mesenteric infarction[59,60]. Intestinal fatty acid binding protein (I-FABP) is a small, cytoplasmic, water soluble protein found abundantly in the epithelial cells of the small intestine. D-lactate has exclusive intestinal source and is produced by colonic bacterial metabolism. Glutathione S-transferase (GST) is a cytoplasmic enzyme and its alpha isoenzyme (alpha-GST) is almost exclusively located in the small intestine and liver. When binding of transition metals (copper, cobalt and nickel) to the N-terminal of Albumin is reduced in hypoxia or acidosis, it is called ischemia modified albumin (IMAL). I-FABP, D-lactate, alpha-GST and IMAL have been shown to be elevated in early AMI[61-63] but at the present time they are not being used in our clinical practice.

As neither symptoms nor Lab tests are specific for the diagnosis of AMI, imaging plays an important role. The recommended first line imaging study for the diagnosis of AMI is computed tomography angiography (CTA) or biphasic multidetector computed tomography of the abdomen and pelvis with IV contrast[64]. CTA is not only rapid, non-invasive, easily available and highly accurate (sensitivity 89%, specificity 99%) in diagnosing mesenteric ischemia but also helpful in detecting the underlying etiology of ischemia[65]. CTA can evaluate the bowel wall, vascular abnormalities and surrounding viscera. CTA may show bowel wall thickening, bowel wall hypoenhancement, intramural hemorrhage, fluid-filled dilated bowel loops, arterial or venous thrombus, decreased filling, narrowing, irregularity, spasm or engorgement of mesenteric vessels, pneumatosis intestinalis, portomesenteric venous gas, or infarction of other viscera[66]. Some of the findings may give a clue to the underlying cause of ischemia: Hypoenhanced bowel wall generally suggests arterial occlusion, bowel wall thickening may indicate MVT and patent SMA with diffuse narrowing of its branches (string of sausages sign) is seen in NOMI[67]. Considering serious and lethal consequences of missed diagnosis of AMI, CTA with intravenous contrast material should be done as soon as possible in patients with suspected AMI irrespective of renal function[68]. Traditional mesenteric angiogram or catheter-based angiogram is the gold standard considering its diagnostic and therapeutic potential but considering its invasiveness, expense and lack of wide availability it is now considered as the 2nd line modality in the evaluation of AMI[69].

Plain X-ray and ultrasound of abdomen have limited role in diagnosing AMI[70]. But plain X-ray abdomen may show free intraperitoneal air if bowel infarction and perforation occur. Mesenteric vessel duplex ultrasound scan (DUS) is useful in the evaluation of AMI. One multicenter study showed that DUS done at the Emergency Department can be helpful (sensitivity 100%, specificity 64%, negative predictive value 100%) in diagnosing and excluding occlusive AMI[71]. DUS is also very helpful in direct evaluation of MVT in the setting of AMI. Although it is specific and can be done at the bedside, it is not as sensitive as CTA or MRA[72]. DUS has also certain limitations that it is operator dependent, not useful in presence of dilated bowel with gas, cannot detect thrombus or embolus beyond the major vessels, and it cannot identify NOMI.

Contrast enhanced MRA is an excellent radiation-free non-invasive test with a sensitivity of 100% and a specificity of 91% in diagnosing AMI. But it is not considered as a practical test in diagnosing AMI considering its long time to do the test and high expense.

Once the diagnosis of MVT is made on imaging, the underlying cause of MVT should be investigated particularly for congenital or acquired thrombophilia. Patients should be evaluated for protein C, protein S and anti-thrombin III deficiency, factor V Leiden mutation, myeloproliferative disorders, homocystinuria, paroxysmal nocturnal hemoglobinuria as well as inflammatory bowel disease when more stable.

CI: There is no Lab test diagnostic of CI. Complete blood count, complete metabolic profile, serum lactate, lactate dehydrogenase (LDH), creatinine phosphokinase (CPK), amylase and coagulation studies are helpful assessing the severity of CI. Low hemoglobin may indicate the amount of colonic blood loss. Leucocytosis, lactic acidosis, hypoalbuminemia, elevated LDH, CPK and amylase may suggest colonic infarction[73]. Plain X-ray abdomen is normal in most of the time but rarely it may show 'thumbprinting' due to submucosal edema/hemorrhage, pneumatosis linearis due to necrosis of the colon wall, portal venous gas, colonic ileus or pneumoperitoneum due to perforation[74]. CT abdomen and pelvis with oral and intravenous contrast is the imaging study of choice to evaluate CI[75]. But as patients with right-sided CI (RSCI) can have large visceral artery occlusion, CTA should be done in case of suspected RSCI or when the diagnosis of AMI needs to be ruled out[76].

Colonoscopy with biopsy within 48 h is the next step to conform the diagnosis of CI. In non-gangrenous IC, colonoscopy may show a highly specific sign like a single linear ulcer running longitudinally along the antimesenteric colonic wall (colon single-strap sign) or non-specific signs like segmental erythema, fragile and edematous colon mucosa, scattered hemorrhagic erosions, scattered petechial hemorrhages with pale areas, bluish hemorrhagic nodules due to submucosal hemorrhage and sometimes mass-like lesions mimicking malignancy[77,78]. In gangrenous IC, colon mucosa will appear as black or gray-green. Histology is nonspecific most of the time. It may show focal crypt drop out, lamina propria and submucosal hemorrhage and edema, hemosiderin laden macrophages in the submucosa, fibrin thrombi in capillaries with infiltration of neutrophils, erosion and granulation tissue hyperplasia[79]. Necrosis and ghost cells *i.e.* preserved cellular outlines with empty cell contents are pathog-

nomonic but infrequently seen. Epithelial loss, presence of acute and chronic inflammatory cells and submucosal congestion can be seen in advanced ischemia.

CMI: Lab tests are generally non-specific but may show anemia, leukopenia, lymphopenia and hypoalbuminemia indicating underlying malnutrition due to CMI. There are various imaging studies available to diagnose CMI. According to American College of Radiology, CTA should be the first-line test to evaluate CMI[80]. Gadolonium-enhanced MRA is highly sensitive in detecting stenosis at the origin of CA, SMA and IMA. One disadvantage of MRA is that it may not be able to evaluate the IMA well[81]. Duplex ultrasound is cost-effective and may play an important role in the diagnosis of CMI. Fasting peak systolic velocity ≥ 275 cm/s in the SMA and ≥ 200 cm/s in the CA can reliably (sensitivity and specificity around 90%) indicate ≥ 70% stenosis[82]. But the study is time consuming and can be unsatisfactory due to bowel gas, obesity, body habitus, complex anatomy, post-operative changes and operator dependency[83]. Catheter-based angiography had been the gold standard for diagnosing CMI for many years[84]. Now-a-days it is done mainly for therapeutic intervention and also for diagnostic confirmation when the results of other imaging modalities are inconclusive.

Management

The main goal of managing AMI is not only prompt diagnosis but also resuscitation of the patient and revascularization of the bowel before irreversible damage occurs. As soon as AMI is suspected, the patient should be given supplemental oxygen and adequate hydration with crystalloid infusion (up to 100 mL/kg) to restore tissue perfusion. Hemodynamic monitoring including mental status and urine output should be done[85]. If the patient still remains hypotensive despite adequate fluid resuscitation, vasopressor pressor agents should be considered. But catecholamines should be avoided as they decrease splanchnic blood flow. Vasopressor agent like dobutamine, low dose dopamine or milrinone can be used to maintain blood pressure as they have less vasoconstrictive effect on mesenteric vessels. Many patients with atrial fibrillation/flutter will need medications to control the ventricular rate. In those cases, medications other than cardiac glycosides should be used as they can also reduce splanchnic blood flow. Any acid-base and electrolyte abnormalities (metabolic acidosis, hyperkalemia) should be corrected. Nasogastric tube should be placed for decompression. Broad spectrum antibiotics and heparin infusion should be started. Both general surgery and vascular surgery teams should be consulted and after resuscitation, laparotomy should be done promptly if the patient has signs of peritonitis (rebound tenderness) indicating bowel infarction. During laparotomy, the whole bowel should be inspected carefully and only the clearly defined infarcted bowel should be resected. According to Bulkley *et al*[86], signs of intestinal viability include normal looking bowl wall without bleeding, pulsatile artery and stimulation of intestinal peristalsis by mechanical factors or heat[86]. If there is any suspicion, intraoperative Doppler ultrasound should be done to find out the presence of Doppler flow over the distal branches of SMA in order to preserve all the viable bowels. Potentially viable bowel should be inspected for 30 min after revascularization. Revascularization technique depends on the underlying pathophysiologic form. In case of SMA embolism, a standard embolectomy is performed by doing a tranverse arteriotomy after clamping the SMA proximal and distal to the embolus[87]. The arteriotomy site is then closed with nonabsorbable interrupted sutures. In case of SMA thrombosis, a longitudinal arteriotomy is done followed by revascularization either by thromboendartectomy with a Fogarty catheter[88] or a bypass procedure. After thromboendartectomy, heparinized saline is used to flush the artery. If thromboartectomy restores blood flow, then the longitudinal arteriotomy is closed in a standard fashion or by patch angioplasty. Retrograde bypass from the iliac artery to the distal SMA using a synthetic graft or a femoral venous graft can also be considered. About 24 to 48 h after restoration of mesenteric blood flow, a second-look laparotomy or laparoscopy should be done to evaluate potentially viable bowel. Assessment can be done by inspection and palpation of the bowel and mesenteric vessels, Doppler ultrasound examination or intraoperative fluorescein administration[89]. Second-look surgery *i.e.* damage control surgery allows identification of necrotic bowel before perforation and contamination occurs, resection of any necrotic bowel, preservation of viable bowel preventing short bowel syndrome and primary anastomosis of viable parts of the bowel.

Over the last 15 years there has been tremendous interest in minimally invasive endovascular treatment of embolic or thrombotic AMI. This should be considered when there is no clinical, serological or imaging evidence of necrosis or infarction of

the bowel wall. Endovascular treatment involves less surgical trauma and less intensive unit care in the elderly patients with multiple comorbidities as compared to open surgical procedures. The endovascular interventions include endovascular embolectomy by transcutaneous mechanical aspiration, percutaneous aspiration thrombectomy, intraarterial pharmacological thrombolysis with infusion of urokinase for 2 to 12 h, and percutaneous transluminal angioplasty with or without stenting[90-93]. Intraarterial infusion of papaverine (30 to 60 mg/dL) is also considered when there is evidence of vasospasm in embolic or thrombotic AMI and the infusion is generally continued for 24 h. Intraarterial bolus followed by infusion of heparin is continued throughout the procedure. But heparin infusion should not be mixed with papaverine (a phosphodiesterase inhibitor) because of the risk of precipitation. After endovascular treatment, patient should receive low molecular weight heparin for 5 d and then anticoagulation should be continued with warfarin. In patients with stenting, in-stent restenosis and high symptom recurrence have been reported. So, if no contraindication exists, patients should be instructed to take daily antiplatelet indefinitely. Although the technical success rate of endovascular treatment in acute arterial mesenteric ischemia can be as high as 100%, intestinal necrosis can occur following the treatment requiring laparotomy and intestinal resection[94]. Other potential complications of endovascular treatment include vascular access-related bleeding, hematoma or pseudoaneurysm formation, and trauma to the vessels leading to arterial dissection or atheroembolization. One study compared endovascular treatment with open surgical treatment. Endovascular treatment group needed less laparotomy (33.3% *vs* 58.3%), had significantly shorter length of bowel resection (88 ± 44 cm *vs* 253 ± 103 cm) and less mortality (16.7% *vs* 33.3%)[95]. A completion angiogram is necessary following endovascular treatment to assess revascularization [96]. Revascularization is required in more than 70% of patients with occlusive AMI while bowel resection alone can be life saving in 30% of patients with occlusive AMI [97]. Hybrid technique (*i.e.* combination of endovascular surgery and open vascular surgery) is also widely used in the treatment of occlusive AMI. Intra-arterial catheter-directed thrombolysis following SMA recanalization by intraoperative retrograde balloon angioplasty, retrograde open mesenteric stenting or open embolectomy during laparotomy has been found to prevent necrosis of ischemic bowel and decrease the occurrence of short bowel syndrome[98].

The main principle of management of NOMI is reversal of mesenteric arterial spasm and restoration of mesenteric blood flow as fast as possible[99]. As soon as the diagnosis of NOMI is made by CTA, patients with NOMI should be resuscitated. The precipitating factors should be addressed to improve mesenteric perfusion. Measures should be taken to correct hypovolumia and hypotension, improve cardiac function in congestive heart failure, treat cardiac arrhythmias, correct metabolic acidosis, initiate broad-spectrum antibiotics, and place a nasogastric tube for gastric decompression. The next step of management depends on the stage of mesenteric ischemia. If the patient has developed signs of bowel infarction, surgical intervention should be done to resect the infarcted bowel and second look surgery should be done to save as much bowel as possible[100]. If there is no sign of bowel infarction, prompt catheter-based mesenteric arteriogram should be done to start continuous intra-arterial vasodilator infusion (papaverine 30-60 mg/h, prostaglandin PGE1 alprostadil 20 mcg bolus followed by infusion 60-80 mcg/24 h) for about 48 h by placing the catheter into the proximal SMA[101]. Frequently arterial vasospasm responds to this direct vasodilator treatment and occasionally within minutes[102]. One study showed that the shorter the time from CTA to direct vasodilator infusion, the better the chance of survival [103].

Treatment of MVT again depends on the presence or absence of bowel infarction. In the absence of bowel infarction, the main stay of treatment is anticoagulation with continuous heparin infusion which has been shown to cause frequent recanalization with better outcome[104]. Systemic thrombolysis with recombinant tissue–type plasminogen activator or transcatheter thrombolysis with urokinase can also be an initial effective treatment for acute superior MVT[105,106]. Anticoagulation with warfarin or direct oral anticoagulants (DOAC) should be continued for 3 to 6 mo in case of reversible causes, and for indefinite period in case of irreversible causes[107]. Patients with bowel infarction should have emergency surgery with resection of the infarcted bowel followed by second look surgery. Transjugular intrahepatic portosystemic shunt can be placed in case of extensive MVT to reduce the portal pressure and to increase the effectiveness of thrombolytic therapy[108].

CI is managed by supportive care in most of the cases in clinical practice. But risk stratification is extremely important in deciding whether supportive care only or surgical intervention is needed. Broadly, CI can be classified as non-gangrenous and

gangrenous forms. But CI can also be classified as mild, moderate and severe disease depending on the presence or absence of certain risk factors associated with poor outcome as proposed by the American College of Gastroenterology (ACG) in 2015 [109]. The risk factors include: (1) Male sex; (2) Abdominal pain without rectal bleeding; (3) Tachycardia (heart rate > 100/min); (4) Hypotension (systolic blood pressure < 90 mmHg); (5) Leukocytosis (white blood cell count > 15000/cmm; (6) Anemia (hemoglobin < 12 gm/dL); hyponatremia (< 136 meq/L); (7) Azotemia (blood urea nitrogen > 20 mg/dL; and (8) High serum LDH level (> 350 units/L). Mild CI: No risk factor associated with poor outcome. Moderate CI: Up to 3 risk factors associated with poor outcome. Severe CI: More than 3 risk factors associated with poor outcome or any of the following: (1) Signs of peritonitis on physical examination; (2) Pneumoperitoneum, portal venous gas or pneumatosis on imaging; or (3) Gangrenous colitis on colonoscopy.

Patients with mild to moderate CI should be managed conservatively with bowel rest, intravenous fluid infusion to improve colonic perfusion and nasogastric tube placement if there is ileus. Any precipitating condition like hypotension or cardiac arrhythmia should be treated. Offending medications like mesenteric vasospastic drugs should be discontinued immediately. Most of the patients with mild to moderate CI improve symptomatically in one to two days and recover completely in one to two weeks[110]. But if the patients continue to have symptoms (abdominal pain, hematochezia) with fever, leukocytosis or develop rebound tenderness, surgical exploration should be considered. Patients with severe CI without perforation or gangrenous colitis also need supportive care but they should have close clinical follow up for development of late complications like chronic IC presenting as chronic diarrhea or ischemic colonic stricture presenting as colonic obstruction. In those cases, patients may need elective segmental colonic resection with primary anastomosis. Patients with gangrenous colitis or colonic perforation should undergo surgical exploration for colectomy.

Patients with CMI should have mesenteric revascularization either surgically or endovascularly. Patient's comorbidities, nutritional status and renal function should be assessed before selecting the revascularization technique. Open surgical procedures include antegrade aorto-mesenteric or aorto-celiac bypass graft, retrograde bypass graft from infrarenal aorta and iliac arteries to distal SMA, endarterctomy and reimplantation of SMA directly into the infra-renal aorta[111-113]. The bypass graft can be synthetic (Polytetrafluoroethylene or Dacron grafts) or autologous (femoral vein or sephanous vein) graft and the type of surgery depends on the location of the stenosis[114]. Open surgical procedures for revascularization are highly successful in symptomatic improvement in 90% to 100% of cases[115] but associated with significant 30 d morbidity (21.1%) and mortality (5.8%)[116]. Percutaneous endovascular treatment (PEVT) involves percutaneous transluminal balloon angioplasty (PTBA) with or without stent placement. Matsumoto *et al*[117] found in a small study that PTBA plus stenting had better technical success and patency rate when compared with PTBA alone[117]. PEVT has high technical success (97%) as well as clinical success *i.e.* resolution of clinical symptoms (90%) in properly selected cases[118]. It is safe with less hospital length of stay, low 30 d mortality (3%) and low complication rate (9%) [119]. There is no prospective randomized control trial comparing open surgical revascularization *vs* PEVT. Experience at the Cleveland Clinic Foundation and Mayo Clinic showed that long term restenosis and recurrence of symptoms were higher in the PEVT group compared to open surgical revascularization group[120,121]. However, PEVT is now-a-days widely used as a first line treatment of choice in high risk patients considering the less invasiveness, morbidity, mortality and success rate. Open surgical revascularization is considered in patients with good clinical condition with acceptable surgical risk considering the less chance of recurrence. So patient selection is very important in deciding which modality of treatment is right for the treatment of CMI. Medical treatment with long-term anticoagulation warfarin is considered for patients who are not candidates for PEVT or open surgical revascularization.

Prognosis

AMI: Despite the advancement in diagnostic modalities and treatment options, the prognosis of AMI remains grave. Adaba *et al*[122] did a meta-analysis of observational studies in patients with acute mesenteric infarction and found that the pooled in-hospital mortality was 63%. Mortality was higher in acute arterial mesenteric infarction (73.9%) and NOMI (68.5% as compared to acute mesenteric venous infarction (44.2%)[122]. Risk factors for increased mortality include advanced age, leukocytosis, high arterial lactate level in the first 24 h of admission, metabolic acidosis, bandemia, sepsis, hypoxia, hyperamylasemia, intramural pneumatosis,

elapsed time between onset of symptoms and surgery more than 24 h, colon involvement, hepatic and renal impairment and multi-organ failure[123-125]. Mortality can be reduced substantially if diagnosis can be made and treatment given before the development of mesenteric infarction[126]. Patients who survive from extensive bowel resection suffer from short gut syndrome and become dependent on total parenteral nutrition. Klempnauer *et al*[127] did a long-term study on those patients and found that their 2-year and 5-year survival rates were 70% and 50% respectively[127].

CI: Most of the patients with CI recover quickly within 1 to 2 wk and the overall mortality is almost 10%. Bad prognostic factors include absence of hematochezia, signs of peritonitis (Blumberg's sign: Rigidity and rebound tenderness), tachycardia, anemia, hyponatremia, right sided colonic involvement and stenosis/stricture seen during colonoscopy[128,129].

CMI: Patients with CMI are at risk of developing acute mesenteric artery thrombosis or embolism that can cause increased morbidity and mortality. Patients with malnutrition due to sitophobia may have to stay at the hospital for prolonged period of time. There are certain prognostic factors associated with bad long-term outcome after open mesenteric revascularization. These include body mass index > 25 kg/m, smoking, peripheral artery disease, hypertensive chronic kidney disease, and open mesenteric bypass with a venous graft[130]. Blauw *et al*[131] did a retrospective study on the impact of revascularization in patients with CMI and found that mesenteric artery revascularization significantly improved their quality of life[131].

CONCLUSION

ISBODI is an important clinical entity we come across in our day-to-day clinical practice. Among the 3 forms of ISBODI (AMI, CMI, CI), CI is the most common form.

CI occurs as a result of non-occlusive ischemia due to small vessel disease in most of the time. Rarely, CI is due to systemic hypotension or vascular occlusion due to atheromatous emboli, vasculitis or vascular spasm. Although CI can occur in a well-known clinical setting, in a vast majority of cases, the risk factor remains unidentified. Patients with CI should be stratified into mild, moderate and severe cases depending on the presence or absence of the risk factors associated poor outcome as proposed by the ACG. Management will depend on the severity of the cases. Patients generally present with lower abdominal pain and hematochezia. Segmental involvements of the splenic flexure, rectosigmoid area and ileocecal region are most commonly seen. Imaging with CT followed by colonoscopy with biopsy clinches the diagnosis. Most of the patients belong to mild to moderate severity and CI carries a benign course in those cases. Surgical intervention is rarely required and indicated for fulminant, gangrenous or perforated CI, non-responders to conservative management, chronic ulcerative IC and ischemic colonic structure. Almost 10% of patients can die from CI. AMI is the most severe and devastating form of ISBODI. It has four pathophysiological forms which include SMA embolism, SMA thrombosis, MVT and NOMI. Irrespective of these pathophysiological forms, in the early phase, patients present with sudden onset of severe abdominal pain without much physical sign. As the mesenteric ischemia progresses to mesenteric infarction, patients develop Blumberg's sign. High clinical suspicion and a focused history are helpful in establishing the diagnosis and suspecting the underlying pathophysiologic form of AMI. Evidence of hemoconcentration, leukocytosis, lactic acidosis and positive D-dimer test may suggest the presence of AMI but the diagnosis is generally confirmed by contrast enhanced CTA. Resuscitation of the patient and revascularization of the bowel are the main aims of treatment of AMI. SMA embolism and SMA thrombosis can be treated by open surgery or PEVT. Combination of both treatments (hybrid technique) is also widely used. NOMI can be treated by intra-arterial vasodilator infusion therapy by placing a catheter into the SMA. In case of MVT, systemic anticoagulation or thrombolytic therapy followed by coumidine or DOAC should be given. If the patient has developed bowel wall necrosis in any form of AMI, open surgery should be done to resect the necrotic bowel and then revascularization should be done. Second look surgery should be done after 24 to 48 h to evaluate the potentially viable bowel and to preserve the bowel as much as possible to prevent short bowel syndrome. CMI occurs when patients develop ischemic symptoms due to stenosis of two of the three major mesenteric arteries. Almost always it develops slowly over the years due to atherosclerotic narrowing of those arteries. Patients generally present with post-prandial

intestinal angina. The classic triad of post-prandial upper or mid-abdominal pain, sitophobia and weight loss is present in less than one quarter of cases. CTA is the diagnostic investigation of choice. Revascularization can be done either surgically or by PEVT. Currently, PEVT is widely used as the first line treatment in high-risk patients and open surgical treatment is reserved for patients with less comoribidities. Revascularization can greatly improve the quality of life in patients with CMI.

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REVIEW

Therapeutic implications of SARS-CoV-2 dysregulation of the gut-brain-lung axis

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Abstract

The emergence and rapid spread of novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused over 180 million confirmed cases resulting in over 4 million deaths worldwide with no clear end in sight for the coronavirus disease 19 (COVID-19) pandemic. Most SARS-CoV-2 exposed individuals experience mild to moderate symptoms, including fever, cough, fatigue, and loss of smell and taste. However, many individuals develop pneumonia, acute respiratory distress syndrome, septic shock, and multiorgan dysfunction. In addition to these primarily respiratory symptoms, SARS-CoV-2

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can also infiltrate the central nervous system, which may damage the blood-brain barrier and the neuron's synapses. Resultant inflammation and neurodegeneration in the brain stem can further prevent efferent signaling to cranial nerves, leading to the loss of anti-inflammatory signaling and normal respiratory and gastrointestinal functions. Additionally, SARS-CoV-2 can infect enterocytes resulting in gut damage followed by microbial dysbiosis and translocation of bacteria and their byproducts across the damaged epithelial barrier. As a result, this exacerbates pro-inflammatory responses both locally and systemically, resulting in impaired clinical outcomes. Recent evidence has highlighted the complex interactions that mutually modulate respiratory, neurological, and gastrointestinal function. In this review, we discuss the ways SARS-CoV-2 potentially disrupts the gut-brain-lung axis. We further highlight targeting specific responses to SARS-CoV-2 for the development of novel, urgently needed therapeutic interventions. Finally, we propose a prospective related to the individuals from Low- and Middle-Income countries. Here, the underlying propensity for heightened gut damage/microbial translocation is likely to result in worse clinical outcomes during this COVID-19 pandemic.

Key Words: SARS-CoV-2; Gut; Microbiome; Lungs; Brain; Therapeutics

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Core Tip: Severe acute respiratory syndrome coronavirus 2 has spread rapidly, infecting and killing millions worldwide. In addition to respiratory symptoms, coronavirus disease 19 (COVID-19) is associated with enterocyte infection leading to intestinal inflammation, gut barrier damage, and microbial dysbiosis exacerbating the systemic inflammatory response. Viral infiltration to the central nervous system from cranial nerve innervation of the lungs and gut can also cause neuroinflammation and degeneration, which further dysregulates gut and lungs. This review summarizes recent findings on COVID-19 pathogenesis in the gut-brain-lung axis and offers therapeutic interventions to improve clinical outcomes.

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INTRODUCTION

Starting from late 2019 and through 2020, a novel coronavirus, the causative agent of coronavirus disease 19 (COVID-19), initiated one of the deadliest global pandemics warranting extensive panic for the underprepared global health system[1-4]. The pandemic has claimed countless lives (approximately 4 million deaths worldwide of 188 million confirmed cases as of July 15, 2021) and presented unprecedented socio-economic losses[5-8]. The primary mode of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) transmission is airborne, with infectious droplets gaining access into the host respiratory tract. Subsequently, standard clinical indicators comprise fever, cough, fatigue, chills, nausea, headache, dyspnea. Additionally, the loss of taste or smell often ensues[9,10]. Coronavirus infections primarily target the upper respiratory tract, but they also manifest in other regions such as the gastrointestinal tract (GIT). Within the GIT, additional symptoms such as lack of appetite, vomiting, abdominal pain, and diarrhea have been observed in some individuals[11,12].

SARS-CoV-2 has been shown to infect GIT cells such as human gut enterocytes[13]. SARS-CoV-2 RNA has also been detected in gastrointestinal glandular epithelial cells [14-17]. During infection, compromised gastrointestinal symptom involvement has worse clinical outcomes and takes longer to recover than those with only respiratory

symptoms[18-20]. Subsequently, patients who test positive for viral RNA in feces have poor viral clearance and prolonged delays for infection resolution[21]. SARS-CoV-2 tropism in the lungs and GIT is supported by abundant cellular viral entry receptors such as the angiotensin-converting enzyme-2 (ACE2). ACE2 exists in abundance on the epithelia of the lungs and small intestine (ileum)[22,23]. ACE2 catalyzes the conversion of angiotensin II into angiotensin 1-7; it is crucial for maintaining gut balance through regulation of amino acid homeostasis, innate immunity, the balance of the intestinal microbiome, and by limiting diarrhea[24,25].

The gut is the largest immune organ and is a crucial interconnecting hub, which synchronously orchestrates multiple physiological, cellular, and organ functions[26]. The gut maintains an anti-inflammatory state that supports the growth of trillions of commensal bacteria, which modulate the function of other organs like the brain, heart, kidney, lungs, and liver while favoring its core functions like digestion, absorption of nutrients, and excretion of waste matter[27-30]. This inter-compartmentalized crosstalk infers the need to develop effective gut-specific therapeutics that could limit systemic pathology during diverse viral infections such as human immunodeficiency virus (HIV), SARS-CoV-2, and other related respiratory viruses.

IMMUNE ARCHITECTURE OF A HEALTHY GUT

The gut is one of the most complex immune organs containing a wide variety of immune cells, including myeloid cells, conventional T cells, innate lymphoid cells (*e.g.*, natural killer cells and specialized intraepithelial lymphocyte populations), and epithelial cells[31]. Homeostasis in a healthy gut is mainly fostered through the sustenance of an anti-inflammatory environment crucial for maintaining tolerance to oral antigens, commensal microflora, and bridging an intact mucosal barrier[32,33]. Commensal microflora competes with pathobionts (potentially pathogenic bacteria which are benign during homeostasis) for nutrients within the gut microenvironment, secrete toxins that favor their predominance, and release metabolites that shape gut immunity by providing appropriate anti-inflammatory signals to other immune cells such as macrophages[31,34].

Critical genera of the microbiome also ferment dietary fiber into short-chain fatty acids that support gut immune responses by sustaining the balance of T regulatory (T reg) cells and activated T cells[35]. The released short-chain fatty acids such as butyrate facilitate CD103+ dendritic cell production of retinoic acid, which in turn aid in the generation of FoxP3+ T reg cells and sampling of an antigen across the gut lumen. Increased frequencies of CD4+ regulatory T cells maintain a basal anti-inflammatory state of the gut. Released acetate also improves the integrity of gut epithelial cells[36,37]. Additionally, lamina propria macrophages are highly phagocytic and engulf any foreign microorganisms that may have breached the intact gut mucosal barrier[38]. The gut epithelial cells secrete anti-inflammatory cytokines such as interleukin 10 (IL-10) and transforming growth factor-β (TGF-β), which promote increased frequencies of T regs that are crucial for immunological suppression and induction of mucosal tolerance[39-41].

SARS-COV-2 INFECTION IN THE GUT

During gut infection, SARS-CoV-2 uses its spike (S) proteins on the virus's surface to directly bind to the ACE2 receptor that is highly expressed on target intestinal epithelial cells (enterocytes and goblet cells). A transmembrane serine protease (TMPRSS2) acts synergistically and cleaves the S protein into S1 and S2 subunits, facilitating the host cell's viral entry[15]. Like other viruses such as rotavirus, norovirus, and enteroviruses, the interferon-stimulated genes are activated, resulting in the reinstatement of an antiviral state and restricted SARS-CoV-2 replication[42-44]. Simultaneously, pro-inflammatory cytokines like IL-6, IL-1, and tumor necrosis factor-α (TNF-α) are also released[44]. Severe enterocyte apoptosis follows as soon as the virus overcomes the interferon-mediated antiviral state[16]. The tremendous loss of intestinal epithelial cells diminishes the gut barrier's integrity and gravely impacts the absorption of nutrients, secretion of mucus, and production of antimicrobial peptides [45]. The elevated secretion of pro-inflammatory cytokines also simultaneously activates macrophages towards pro-inflammatory states, which involves the increased surface expression and later shedding of markers such as CD14 and CD163 that are critical for detecting microorganisms and limiting inflammation[16].

A recent unpublished study that comprehensively investigated changes in gut dynamics during SARS-CoV-2 infection shows that there were increases in permeability of gut tight junctions as estimated using zonulin, microbial translocation, lipid-binding protein, and myeloid activation using soluble CD14[46]. Gut barrier damage, microbial translocation, and myeloid cell activation worsened as the disease transitioned into more severe states. All this was heralded when a two-fold increment in tight junction permeability was observed in individuals who died *vs* COVID-19 survivors. Unsurprisingly, alterations in gut homeostasis positively correlated with elevated IL-6, thus confirming increased inflammation as a conduit for loss of gut barrier integrity[46]. Lastly, progressive increments of disrupted citrulline metabolism accompanying the severity of SARS-CoV-2 indicate ongoing microbial dysbiosis and impaired intestinal function[47].

GUT INFLAMMATION AND SARS-COV-2

An optimal balance between T regs and inflammatory T helper 17 (Th17) cells is maintained within a healthy gut. As inflammation occurs, this balance is tipped as pro-inflammatory bacteria are favored over commensals[37]. These pro-inflammatory bacteria induce the secretion of cytokines like IL-6 and IL-17 that select for increased frequencies of Th17 pro-inflammatory CD4+ T cells and susceptibility to inflammatory conditions such as inflammatory bowel disease (IBD)[37].

An increased expression of SARS-CoV-2 receptors during other inflammatory conditions has been suggested to affect disease pathogenesis. During IBD and Crohn's disease (CD), epithelial ACE2 expression is elevated in the colon and rectum while diminished in the ileum[48]. As anticipated, gut dysbiosis occurs as pathobionts outcompete commensal bacteria, and macrophage populations transition towards inflammatory states where they secrete increased amounts of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-12[49,50]. The resultant inflammatory damage leads to the destruction of tight junctions within the gut barrier. This later leads to leakage of microbial contents into the systemic circulation. Microbial translocation then ensues and later fuels chronic immune activation[49].

Several studies have investigated the co-occurrence of IBD and COVID-19-related illnesses[51-53]. Here, we discuss the possibility of leveraging IBD therapeutics for treating COVID-19-related symptoms. Current IBD treatments can be divided into two broad categories: (1) Non-biologic; and (2) Biologic therapeutics. Examples of non-biologic therapeutics include small molecules corticosteroids, thiopurines, and 5-aminosalicylates. Each of these therapeutics non-specifically targets multiple inflammatory processes.

On the other hand, biologic therapies target specific inflammatory mediators in the gut to modulate their ability to induce signal transduction[41]. Current biologics used to treat IBD include the monoclonal antibodies infliximab, ustekinumab, and vedolizumab. Infliximab and ustekinumab target pro-inflammatory cytokines TNF-α and IL-12/IL-23, respectively. Since these cytokines are not specific to intestinal inflammation, antibodies that inhibit their action can act throughout the periphery. More specific therapies target IBD like vedolizumab function by blocking the gut homing receptor α4β7 integrin, thereby blocking inflammatory lymphocyte trafficking resulting in reduced inflammation[41,54,55]. Similarly, the biologic natalizumab, an anti-α4 integrin antibody, is also used to treat Crohn's disease, albeit less frequently due to an increased risk of progressive multifocal leukoencephalopathy (Table 1)[56].

Several studies have suggested that IBD non-biologic therapies may worsen clinical outcomes during COVID-19[57-59]. For example, 5-aminosalicylate and its prodrug form sulfasalazine have increased the risk of SARS-CoV-2 infection and increased the risk of hospitalization and mortality[57-59]. Other immunomodulators include thiopurines, calcineurin inhibitors, and methotrexate. When grouped in meta-analyses, these immunomodulators were also associated with an increased risk of infection, hospitalization, and mortality during SARS-CoV-2 infection[57-59]. The data for corticosteroids is more complicated[57-59]. Owing to their nature as non-selective hormone analogs, corticosteroids target gut inflammation non-specifically, resulting in a dampening of the inflammatory response beyond the intestines. This dampened immune response can therefore inhibit the clearance of replicating virus and prolong the duration of infection. Corticosteroids may further negatively impact clinical outcomes by increasing the pro-thrombotic response to SARS-CoV-2, exacerbating already frequent clotting in intrinsically vascular disease. Several case studies have linked this hypercoagulable state in COVID-19 to worsening in the gut, including

Table 1 Comprehensive list of available gut-based and vagus nerve therapies that could be leveraged to limit the severity of coronavirus disease 19 infection

Therapy	Target	Impact	Clinical outcome
Infliximab[57,58]	TNF-α	Reduced pro-inflammatory response induction and leukocyte migration	Reduced infection rate, symptoms, hospital rate, and mortality
Ustekinumab[57]	IL-12/IL-23	Blocks T cell activation	Possible improved outcome (pooled with other biologics)
Vedolizumab[54,57]	α4β7 integrin	Reduced leukocyte trafficking to gut and associated inflammation	Possible improved outcome (pooled with other biologics)
Corticosteroids[57,58,63]	Glucocorticoid and mineralocorticoid receptors	Reduced inflammatory response	Better or worse clinical outcomes, depending on the timing
Microbiome[176]	Butyrate production	Improved gut barrier integrity and decreased microbial translocation	Reduced inflammatory response
Vitamin D[70,72,74]	Th17 cells	Reduced gut and systemic inflammation	Reduced infection risk and enhanced clinical outcomes
Nicotine and related agonists [142,152,153]	α7nAChR	Reduced Inflammatory Response	Reduced infection rate
Vagus nerve stimulation[151]	α7nAChR	Increased acetylcholine release	Reduced inflammation

TNF-α: Tumor necrosis factor-α; IL: Interleukin; Th17: T helper; nAChR: Nicotinic acetylcholine receptor.

ischemic colitis, potentially aggravating an already disrupted gut homeostasis[60,61]. Therefore, it is unsurprising that studies to determine the impact of IBD therapy on COVID-19 have not been pursued. Nonetheless, corticosteroids for IBD are typically administered long-term and precede SARS-CoV-2 infection. Corticosteroids, particularly dexamethasone, reduce mortality in hospitalized SARS-CoV-2 patients receiving oxygen and reduce intensive care unit hospitalization length when given at moderate doses over a short period (6 mg for up to 10 d)[20,62]. In this context, dexamethasone is typically given after the body has begun clearing the virus but before the worst symptoms of COVID-19 due to cytokine storm arise[63]. The difference in infection rate and clinical outcomes for patients receiving treatment before infection suggests that timing is critical for optimizing corticosteroid use.

In addition to modulating immune responses directly, non-biologic medications may influence COVID-19 pathogenesis in other ways. For instance, people receiving non-biologic medications such as corticosteroids, thiopurines, and 5-aminosalicylates have reduced ACE2 and TMPRRS2 expression on enterocytes in the inflamed colon rectum, but not the ileum[48]. This suggests that despite the adverse clinical outcomes with these medications, they may reduce productive gut epithelial infections in people living with IBD. However, these drugs did not cause changes to receptor expression in the uninflamed gut[48]. In contrast, some biologics used to treat IBD have been mostly, though not exclusively, linked to improved clinical outcomes during SARS-CoV-2 infection, offering novel therapeutic interventions. Early studies have shown that the patients administered biologics did not have an increased risk of COVID-19, despite being considered immunocompromised[64,65]. Individual case studies demonstrate that targeting TNF-α with infliximab promises to improve pulmonary symptoms related to COVID-19[66]. Further, an early study grouping multiple biologic therapies showed that clinical outcomes for individuals taking these biologics were five times less likely to be diagnosed with a SARS-CoV-2 infection[67]. However, this analysis was limited due to the small sample size and inability to separate the specific therapies.

A more recent meta-analysis, again grouping different biologics (anti-TNF-α, ustekinumab, and vedolizumab), demonstrated that their use was associated with a risk ratio of hospitalization 0.34 [95% confidence interval (CI) 0.19-0.61], need for intensive care unit 0.49 (95%CI 0.33-0.72), and mortality 0.22 (95%CI 0.13-0.38), suggesting a substantial improvement in clinical outcome[57]. However, one concern is that because people treated with biologics have such muted symptomology, they may serve as silent carriers of SARS-CoV-2[68]. In addition to reducing symptoms, infliximab has also been shown to reduce the expression of ACE2 in the colon, potentially limiting the productive infection of the gut[48]. Because of these findings, a continued study of these monoclonal antibodies is warranted. Luckily, several clinical trials are currently underway, including anti-TNF- α therapies infliximab and

adalimumab.

In addition to prescription therapeutics, other recommendations for IBD can also be applied to SARS-CoV-2 infection. COVID-19 pathogenesis has been characterized primarily by dysregulation of the systemic pro-inflammatory response of Th17 cells, particularly during the cytokine storm. This includes decreases in CD4+ T cells expressing Th17 markers CCR6 and CD161, increasing senescence and exhaustion markers CD57 and PD-1[69]. The total number of IL-17A-producing CD4+ and CD8+ T cells was significantly increased despite these findings. These studies suggest that targeting IL-17 may improve clinical outcomes for COVID-19 patients[69].

Further, early during the pandemic, low circulating vitamin D became a known risk factor for SARS-CoV-2 infection, and severe pathogenesis, including associations with significantly higher pro-inflammatory cytokines, including IL-6[70,71] was noted. For this reason, vitamin D supplementation has been recommended for high-risk individuals[70]. Oral vitamin D supplements are absorbed by the small intestine, where they may modulate local Th17 cells, including reduced production of IL-17[72]. In clinical practice, vitamin D is frequently recommended for patients prescribed corticosteroids to manage their IBD symptoms to prevent bone density loss[73]. It is also recommended to reduce or protect against IBD onset of symptoms, in part by suppressing IL-17 production[72]. Further, vitamin D has been linked to increased expression of tight epithelial junctions and blocking apoptosis in the intestine, strengthening gut integrity and preventing bacterial translocation[74,75]. However, whether dampened COVID-19 pathogenesis is linked to inhibition of pro-inflammatory Th17 cells or by a different mechanism remains unknown.

One final concern regarding SARS-CoV-2 during IBD has been the safety and efficacy of vaccination with current therapeutics, many of which are immunosuppressive because so far, phase III clinical trials have excluded patients with IBD[76,77]. Previous studies have found IBD patients treated with anti-TNF-α therapies have a muted response when administered other vaccines (pneumococcal, influenza), leading to further questioning of efficacy[76,78,79]. Despite these concerns, the benefits of vaccination outweigh many of the risks of SARS-CoV-2 infection, and vaccination is likely beneficial for most patients with IBD, even if receiving immunosuppressive therapies[76,80,81]. However, future studies are needed to determine efficacy for this population.

SARS-COV-2 INFECTION RESULTS IN MICROBIAL DYSBIOSIS IN THE GUT

SARS-CoV-2-related microbial dysbiosis manifests the enrichment of pathobionts such as *Ruminococcus gnavus*, *Ruminococcus torques*, and *Bacteroides dorei* (*B. dorei*) *Bacteroides vulgatus*. These bacteria have also been implicated in IBD and ulcerative colitis pathogenesis, further strengthening their role in gut dysfunction[82,83]. SARS-CoV-2 dysbiosis was also accompanied by loss of crucial bacteria that have been previously implicated in the lowering of inflammation, such as *Bifidobacterium adolescentis, Faecalibacterium prausnitzii* (*F. prausnitzii*), *Collinsella aerofaciens,* and *Eubacterium rectale* (*E. rectale*)[84] (Figure 1). Another study highlighted that *Clostridium hathewayi* (*C. hathewayi*), *Clostridium ramosum* (*C. ramosum*), and *Coprobacillus* were linked to COVID-19 severity[85]. Unsurprisingly, these microbial signatures (*C. hathewayi, C. ramosum, and Coprobacillus*) have also been associated with pathogenic/inflammatory gut conditions such as diarrhea, colitis, IBD, and overall inflammation[86-88]. This situation's gravitas is further supported by observations made in a separate study that showed that the inflammatory species, *Proteobacteria,* was reported in the guts of COVID-19 patients[89].

Alternatively, several anti-inflammatory bacteria, including *F. prausnitzii, B. dorei, Bacteroides thetaiotaomicron*, *Bacteroides massiliensis*, and *Bacteroides ovatus* were linked to improved clinical outcomes in SARS-CoV-2 patients. Remarkably, *Bacteroides* were reported to down-regulate ACE2 expression in the gut[85]. The fecal microbiome has also been determined to be dysbiotic for periods as long as 30 d after clearance of SAR-CoV-2 infection, further contributing to the extensive list of health complications reported in COVID long haulers who never wholly recover and recuperate to a healthy state[84,90]. To this effect, it was noted that microbiomes of recovered patients were enriched in *Bifidobacterium dentium* and *Lactobacillus ruminis* species and reduced *E. rectale*, *Ruminococcuss bromii*, *F. prausnitzii*, and *Bifidobacterium longum*[84]. Therapeutics involving antibiotics to stabilize the gut microbiome by eliminating SARS-CoV-2 associated pathobionts revealed that these regimens did not affect the

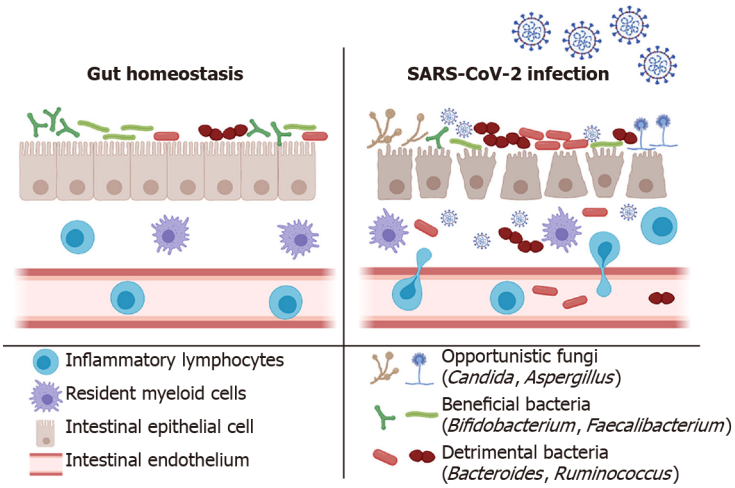


Figure 1 Severe acute respiratory syndrome coronavirus 2 infection induces microbial dysbiosis and intestinal inflammation. Severe acute respiratory syndrome coronavirus 2 infections of intestinal epithelial cells result in a pro-inflammatory immune response leading to infiltration of inflammatory lymphocytes and disrupting the gut barrier. This disruption to homeostasis allows overgrowth of detrimental bacteria resulting in dysbiosis, and the impaired gut barrier facilitates the translocation of bacteria exacerbating the inflammatory response. Additionally, opportunistic fungal infections have been found in some patients, further contributing to the dysfunction. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

recovery of the microbiome. Therefore, these findings negate this approach as a possible future gut microbial rejuvenation strategy[84]. In contrast, previous studies have shown that probiotics improve immune responses to other respiratory tract viral infections such as Influenza and Rhinoviruses[91,92]. Also, in patients with severe COVID-19 disease who cannot ingest probiotics, the use of butyrate enemas could act as adjunctive therapy for repairing intestinal epithelial cell wall damage, lowering chronic immune activation promoting gut-brain axis function[93].

Analysis of the fungal mycobiome using deep shotgun metagenomic sequencing revealed significant proportions of opportunistic fungal pathogens such as *Candida albicans, Candida auris,* and *Aspergillus flavus* were enriched in the gut microbiota of COVID-19 hospitalized patients[94]. Similarly, separate studies have detected *Aspergillus* in respiratory tract secretions and tracheal aspirates of patients with COVID-19[95,96]. Unsurprisingly, in patients with *Aspergillus* in their fecal microbiomes, the cough was noted as one of the significant symptoms that emphasized intricate systemic distortion of the gut-lung axis[97]. In such cases, antifungal medications should be considered.

THE SARS-COV-2 INFECTION AFFECTS THE HOMEOSTASIS OF THE GUT-LUNG AXIS

The gut-lung axis has been proposed to be a bi-directional conduit by which each organ can modulate the function and inflammatory state. This includes the production of metabolites such as short-chain fatty acids (SCFAs) by the gut microbiome, lymphocyte trafficking from the lungs or the gut into the periphery where they redirect systemic immune responses, and indirect neuronal innervation *via* the central nervous system (CNS) and cranial nerves bridging each organ[98,99]. The bi-directionality of the gut-lung axis, while capable of promoting beneficial communication, as noted, also represents an avenue for disruption in immune regulation. Changes in the composition and function of the gastrointestinal flora operate to effect change in the respiratory tract through the mucosal immune system, while reciprocally, disturbances to the respiratory tract flora consequently affect the digestive tract through immune regulation[100]. Infection from SARS-CoV-2 causes effector CD4+ T cells to reach the small intestine by accessing the C-C chemokine receptor type 9 (CCR9) *via* the gut-lung axis resulting in intestinal immune damage and diarrhea[101]. A study related to the influenza virus demonstrated that lung-derived CCR9+CD4+ T cell levels were increased following viral infection[102]. Ye *et al* [101] summarized that the small intestinal epithelium can express CCL25, which actively recruits CCR9+CD4+ T cells to the small intestine, resulting in intestinal immune damage and polarization of Th17 cells in conjunction with overproduction of

IL-17A. This, in turn, leads to neutrophil recruitment, thus establishing diarrhea onset and other gastrointestinal symptoms during SARS-CoV-2 infection. Generally, COVID-19 patients presenting with gastrointestinal symptoms (> 20% of cases) are more likely to be complicated by acute respiratory distress syndrome (ARDS) and are more prone to fatigue, headache, and cough[101]. Another pathway that may be involved in the severity of ARDS during SARS-CoV-2 infection is the C5a-C5aR1 axis. Proportionate to the severity of ARDS, soluble levels of C5a were found to be increased, and C5aR1 receptors were reported with high expression levels in the blood and pulmonary myeloid cells; furthermore, in C5aR1 knock-in mice, acute lung injury was prevented by the introduction of anti-C5aR1 therapeutic monoclonal antibodies suggesting that this axis could be used to limit lung inflammation[89]. Given that ACE2 is highly expressed in the gastrointestinal tract, specifically intestinal epithelial cells, the esophagus, and the lungs, this may make it a target organ for SARS-CoV-2 infection[103]. Therefore, probiotics offer a favorable potential treatment addressing the gastrointestinal symptoms in COVID-19 by operating to sustain the healthy balance of the gut microecology and protect the respiratory tract from bacterial infections[100]. ACE2, while present across different tissue types, is most prominent in the lungs, which highlights the importance of understanding the network involved to combat the hypersensitivity of the receptor in this tissue, which may lead to worse respiratory illness.

Another branch that extends from the lung-gut axis includes the lung-brain conduit, which proposes to connect severe neurological dysfunction and injury with associated lung injury. In general, CoV-induced inflammation and oxidative stress may represent a crucial mechanism for the onset of neurological symptoms. SARS-CoV-2, in particular, has been shown to have the neuroinvasive potential[104]. The vagus nerve innervates the lung's distal airway, and upon stimulation, nerve endings release acetylcholine (ACh), which functions to regulate lung infection[105] (Figure 2). ACh secreted from neuronal and non-neuronal sources acts to decrease inflammation, and using an Influenza A model, a previous study showed that viral infection caused an influx of cholinergic lymphocytes while inhibiting ACh synthesis resulted in extended pulmonary inflammation, increased inflammatory macrophage activation, and delayed tissue repair (Reviewed in[106]).

Similarly, it was demonstrated that a deficiency to the α7 nicotinic ACh receptor (nAChR) acts to worsen lung infection, inflammation, and injury while increasing circulatory levels of pro-inflammatory cytokines. Additionally, α7 nAChR activation by GTS-21, which enhances the phagocytic ability of hyperoxic macrophages, is a prominent method for improving bacterial clearance and reducing lung injury[107], suggesting an involvement of lung-gut-brain connection. During infection, viruses may breach the CNS by various routes, including the vasculature, olfactory and trigeminal nerves, or the lymphatic system. However, the direct mechanism that SARS-CoV-2 utilizes is unknown[108]. One hypothesis is that SARS-CoV-2 enters the CNS by breaching the olfactory route, thereby circumventing the blood-brain barrier (BBB). More than 80% of COVID-19 patients presented with gustatory and olfactory impairments lending support to this access point[104].

Furthermore, in a cohort of 214 COVID-19 patients, 36.4% developed neurologic symptoms ranging from acute cerebrovascular diseases, consciousness impairment, and skeletal muscle symptoms[109]. As reviewed by Nuzzo and Picone, another study used a cellular model to demonstrate that respiratory syncytial virus infection propagates reactive oxygen species (ROS) production transitively, creating oxidative stress that supports the idea that lung inflammation may be a determinant of systemic oxidative stress[104]. The brain, a primary tissue for metabolizing oxygen, is particularly vulnerable to ROS, and SARS-CoV-2 could generate enough ROS to cause brain injury.

In addition to the host immune system's direct mutual interaction in the gut-brain-lung axis, commensal and pathogenic bacteria in the gut microbiome can also influence COVID-19 pathogenesis. SCFAs such as acetate, butyrate, and propionate, being the most prominent immunomodulatory metabolites, maintain and reinforce intestinal epithelial integrity to decrease inflammation in the gastrointestinal tract and respiratory tract by increasing differentiation and fortifying against tight junction permeability[110]. During SARS-CoV-2 infection, increased permeability was associated with clinical worsening, including multiorgan failure[111]. SCFAs produced by the microbiota influence the inflammatory state of the host's epithelial cells, innate and adaptive immune cells directly or indirectly, and utilize the mesenteric lymphatic system for translocation of intact bacteria, their fragments or metabolites through the intestinal barrier, thereby modulating the lung immune response upon entry to the systemic circulation[112,113]. Additionally, SFCA butyrate suppresses intestinal

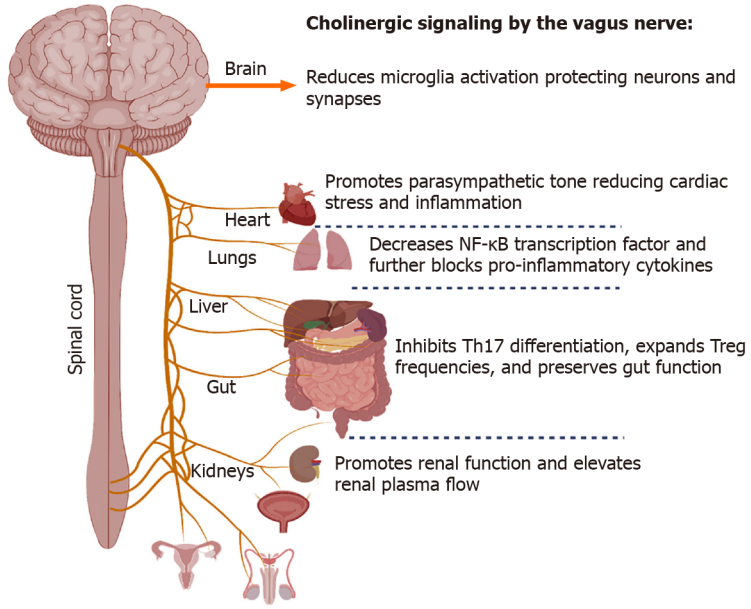


Figure 2 Role of acetylcholine on immune micro-environments in diverse tissues including the brain, heart, lungs, and gut. Cholinergic signaling can be exploited to reduce microglia activation in the central nervous system, lower systemic inflammation, and promote optimal organ function in diseased states such as severe coronavirus disease 19 infection where systemic dysfunction occurs.

inflammation by inhibiting histone deacetylase (HDAC) activity. Inhibition of HDAC enhances the mTOR-S6K pathway required for T cell differentiation and cytokine expression and enhances histone 3 acetylation at Foxp3 Locus to allow transcription to occur and Treg cell differentiation[114,115] Further, GPR43 activation by butyrate can inhibit NF-κB activity and stimulate anti-inflammatory signaling, including IL-8 and IL-10[116].

Previous work on other respiratory viruses also documents disruptions to the gut microbiota. Interestingly, SARS-CoV-2 has been reported to deplete the levels of short-chain fatty acid-producing bacteria such as *Parabacteriodes merdae, Bacteriodes stercoris,* and *Lachnospiraceae bacterium*[117]. Thus, respiratory influenza can indirectly induce intestinal immune injury and alter gut microbiota, resulting in the outgrowth of *Enterobacteriaceae* and reduction of *Lactobacilli* and *Lactococci*[113]. In the influenza-related lung response in mice, intestinal TLR activation is required for NF-κB-dependent pathways for the innate immune response. In this case, disruptions of the microbiota were unrelated to the influenza virus and mediated by Th17 cells[118].

Similarly, other respiratory infections, such as a respiratory syncytial virus (RSV), distort the gut microbiome's balance by depleting *Lachnospiraceae* and *Lactobacillaceae* families while enriching *Bacteroidaceae*[119]. Experimental studies designed to administer high fiber diets favor the enrichment of short-chain fatty acid butyrate-producing bacteria, thus promoting the interferon antiviral state within the lungs of RSV-infected individuals[120]. In turn, depletion of the gut microbiome has been shown to hinder optimal alveolar macrophages' optimal functionality by reducing oxidative respiratory burst capacity and later diminishing their bactericidal effects [121].

IMPACT OF SARS-COV-2 INFECTION ON THE BRAIN-GUT AXIS

The gut microbiome has been shown to directly modulate multiple brain functions such as immune modalities like inflammation and neuroendocrine states like mood, stress, anxiety, and memory functions[122-125]. Within the gut, two plexuses comprise the enteric nervous system (ENS): one at the submucosa and the other associated with the muscularis propria. Together, these two plexuses sense the inflammatory and local microbial milieu. The ENS can regulate many aspects of CNS health and behavior through the production of inflammatory mediators, hormones, and direct neuronal innervation of the vagus nerve from the medulla oblongata[126,127]. The release of short-chain fatty acids by gut commensal bacteria improves the BBB's integrity, reduces inflammation signaling in microglia/astrocytes, and enhances neurogenesis within neurons. Collectively, this promotes reduced aging, better memory, and

improved cognitive development[128].

During gut inflammation, as exemplified with IBD, distorted microbiota populations drive mucosal myeloid cells' activation and subsequent release of pro-inflammatory cytokines that sustain epithelial gut damage. In turn, this activates a cascade of gut pain sensory pathways, dysregulates the enteric nervous system, and simultaneous gut-brain dysfunctions[129-132]. Besides distortion of the lung-gut axis, SARS-CoV-2 dysbiosis of the microbiome could also negatively impact other organs such as the brain. SARS-CoV-2 diminishes crosstalk with the brain by depleting crucial short-chain fatty acid-producing bacteria within the gut microbiome[117] (Figure 3). Furthermore, SARS-CoV-2 has been shown to gain access to the brain by crossing the neural–mucosal interface in olfactory mucosa, where the olfactory mucosal, endothelial, and nervous tissue are closely intertwined[133]. After this, SARS-CoV-2 localizes within the medulla, where respiratory and cardiovascular activities are controlled[133-135]. Thus, this could account for the neurological symptoms such as loss of smell and taste, persistent headaches, confusion, cerebrovascular disease, muscle pain, ataxia, seizures, and dizziness observed in COVID-19 patients[136]. The CNS also supports gut function making the gut-brain axis bi-directional[137,138]. However, the impact of SARS-CoV-2 pathology within the CNS on the direction of gut functions such as peristalsis, modulation of gut immune responses, and directing digestion is yet to be fully comprehended[139].

NICOTINE RECEPTORS, POSSIBLE THERAPEUTIC TARGETS FOR LIMITING SARS-COV-2 DAMAGE IN THE GUT AND BRAIN

Several epidemiological studies from the United States, Europe, and south and east Asia have concluded that tobacco smokers have a lower risk of SARS-CoV-2 infection [140-144]. Additionally, smokers may also have a reduced risk of significant symptoms once infected with SARS-CoV-2, despite typically experiencing increased morbidity and mortality during other respiratory infections[142]. Nicotine is the receptor agonist for nAChRs that contribute to tobacco smoke's addictive and psychoactive properties. In contrast to exogenous nicotine, the endogenous agonist for nAChRs, ACh is produced by neurons and lymphocytes, an essential neuro-immune modulator. The α 7nAChR, in particular, regulates the cholinergic anti-inflammatory response (CAIP) [145]. During acute inflammation, efferent signals from the CNS induce the release of ACh at the enteric nervous system, where they modulate T-cell dynamics, including the induction of Tregs and inhibition of Th17 cells and suppression of local inflammation, including the releases of TNF-α, IL-1β, and HMGB1[146,147]. One hypothesis for nicotine's inhibitory effects is that exogenous α7nAChR agonists recapitulate the effects of ACh release by the vagus nerve, thereby reducing cytokine storm risk both locally at the inhalation site (the lungs) and systemically, including in the gut. This mechanism may be a likely reason nicotine improves ulcerative colitis symptoms[146, 148]. The argument for cholinergic modulation is made even more compelling when considering that neither smoking nor nicotine consumption decreases ACE2 or TMPRSS2 and may increase their expression in the lower airways[149,150]. COVID-19 patients should not be encouraged to smoke or consume nicotine. However, alternative methods of modulating the CAIP have been suggested. Vagus nerve stimulation (VNS) delivers electrical impulses to the left vagus nerve *via* a small device implanted below the collar bone currently used to treat treatment-resistant epilepsy and major depressive disorder. However, less-invasive transcutaneous auricular VNS is being developed. Although more extensive clinical studies are ongoing, case studies have indicated possible benefits in reducing plasma IL-6 levels when using these devices[151]. Alternatively, α7nAChR agonists or acetylcholinesterase inhibitors have been proposed to reduce systemic inflammation[152,153].

A recent report describing ACE2 and TMPRSS2 expression during chronic vagus nerve stimulation showed no changes in either protein or enterocytes[154], although each gene was strongly correlated with CHRNA7 gene expression RNA-seq[154]. Another report separately described how the spike glycoprotein's receptor-binding domain on SARS-CoV-2 possesses significant structural homology with α-bungarotoxin, a neurotoxin produced by kraits that target α7nAChR, the product of CHRNA7 translation[155]. In their discussion, the authors suggest this homology blocks normal cholinergic signaling by competitive inhibition leading to an excessive pro-inflammatory response that cannot be alleviated by vagal signaling. If that is the case, the co-expression of CHRNA7 and ACE2 with TMPRSS2 would mean that infected gut epithelial cells are producing viral particles that inhibit cholinergic

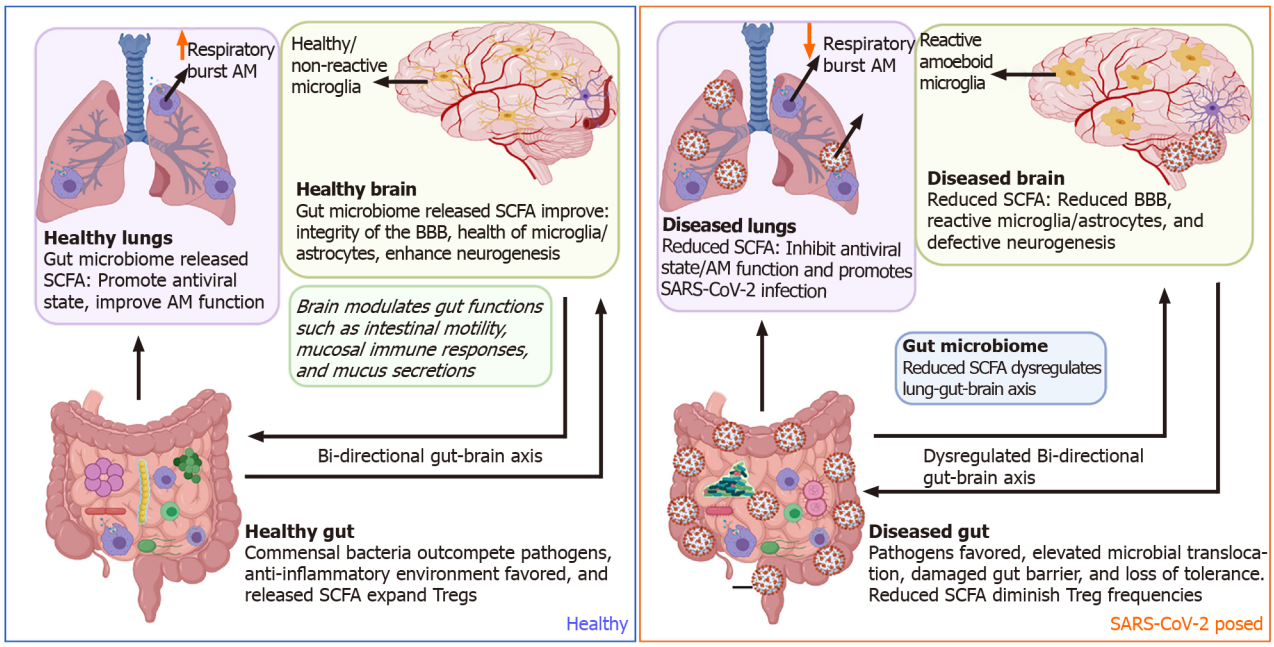


Figure 3 Potential mechanisms by which severe acute respiratory syndrome coronavirus 2 dysregulates the gut-brain axis. Side by side comparisons of the gut-brain-lung axis in a healthy state *vs* a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) exposed state. In a healthy state, commensal bacteria outcompete pathogens within the gut micro-environment leading to a predominantly anti-inflammatory state. Peptides released by commensal gut bacteria support optimal brain and lung function. During SARS-CoV-2 infection, gut microbial dysbiosis dysregulates gut, lung, and brain function. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; BBB: Blood-brain barrier; AM: Alveolar macrophages; SCFA: Short-chain fatty acids; T-regs: T regulatory cells.

signaling on themselves. A loss of cholinergic signaling fails to block HMGB1, allowing increased microbial translocation at the gut barrier exacerbating inflammatory responses[156]. Targeting this interaction on colonocytes may offer considerable protection from pathogenesis, including the cytokine storm. A similar correlation between ACE2 and CHRNA7 expression has been found on airway epithelial cells, suggesting these cells specifically may be the mechanism by which nicotine modulates infection rates and pathogenesis when endogenous ACh fails[157].

It should be noted that in addition to a disruption to its anti-inflammatory functions, the vagus nerve may also contribute to the COVID-19 pathology in other ways. During Parkinson's disease, α-synuclein aggregates travel from the gut to the CNS in the vagus nerve *via* retrograde axonal transport[158]. The innervation of both the lungs and gut, where significant viral replication occurs, may allow a similar direct transport to the CNS[159]. This hypothesis is corroborated by the recent reports, which showed that the brain stem has an increased inflammatory response during COVID-19 compared with other brain regions. Where cranial nerves IX and X (glossopharyngeal and vagus nerves) meet, the medulla oblongata, in particular, has a higher viral load than the rest of the CNS[160]. The axonal and synaptic loss at the dorsal motor nucleus and nucleus solitarius near this region may be contributing to the loss of autonomic control of respiratory function, cardiac arrhythmias, and gastrointestinal symptoms like diarrhea and vomiting[161,162]. Complicating the role of nicotine in COVID-19 pathogenesis, its administration can inhibit Th17-driven neuroinflammation, thereby possibly protecting these regions in smokers[163]. A better understanding of this process, if it exists, could contribute significantly to minimizing neuropathogenesis during SARS-CoV-2 infection.

CONCLUSION

SARS-CoV-2 infection in the gut leads to alterations in gut microbiota with depletion of bacterial genera that produce crucial metabolites such as short-chain fatty acids that sustain optimal brain function. Future studies focusing on using probiotics to replenish commensals' dwindling populations as a strategy to reverse gut dysbiosis while maintaining the gut-brain axis during COVID-19 are needed[164]. Furthermore, additional efforts should be applied to the testing strategies currently used to manage inflammatory conditions affecting the gut for their potential in the direction of

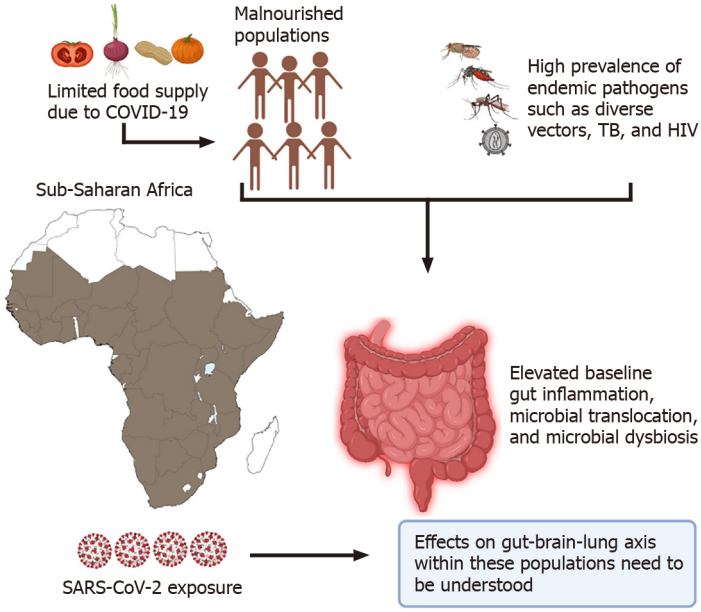


Figure 4 Elevated baseline gut inflammation observed in Sub-Saharan African individuals could lead to severe coronavirus disease 19 associated dysregulation of the gut-brain-lung axis. Continuous exposure to various endemic pathogens such as human immunodeficiency virus, tuberculosis, multiple vectors like tsetse flies, and several mosquitoes, in addition to low diets, set the ground for high levels of gut dysbiosis within the entire community populations. Additional studies focused on evaluating the effects of baseline gut dysbiosis on coronavirus disease 19 infection are highly warranted. SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; COVID-19: Coronavirus disease 19, HIV: Human immunodeficiency virus; TB: Tuberculosis.

COVID-19 associated dysbiosis, gut damage, and microbial translocation. Targeting vagal stimulation and diverse nicotinic receptors could offer lower organ inflammation and confer systemic protection across the gut-brain and lung organs.

Lastly, special consideration should be given to populations worldwide where there is a high incidence of coinfections such as HIV and tuberculosis (TB) that target the gut early on in infection, cause dysbiosis and lead to microbial translocation[165-167]. In the world's poorest regions like Sub-Saharan Africa, most individuals are continuously exposed to endemic pathogens like helminths and TB that could lead to elevated baseline levels of gut damage, immune activation, and possible microbial translocation [168-171]. Recently, Bhaskaran *et al*[172] analyzed data from 27480 individuals co-infected with HIV within the United Kingdom. Their data revealed that people living with HIV had a higher risk of COVID-19 death than those without HIV, after adjusting for the age and sex with a hazard ratio (HR) of 290 (95%CI 0.196-0.430; $P < 0.0001$). However, the absence of testing, limited case reporting, and poor health care limits our understanding of the SARS-CoV-2 pandemic on this continent[172].

The sudden lifestyle changes imparted by the pandemic within these populations have tremendously strained their chances of survival. In countries where restrictions or lockdowns are enforced, most individuals from these regions rely on the government to supply free food rations[173]. However, the low quality of this freely distributed food fuels the already highly prevalent risk of malnutrition within these populations[174]. As we have illustrated, limited dietary access within these communities could further aggravate gut dysbiosis leading to severe systemic disorientation during SARS-CoV-2 infection. The complexity of these variables, in addition to limited health service delivery and governmental support[175] could exacerbate gut-brain disruptions within diverse populations in Sub-Saharan Africa (Figure 4).

In these trying times of the COVID-19 pandemic, the connotation of a healthy gut-healthy mind could translate into one's ability to orchestrate a meaningful immune response that could limit SARS-CoV-2 infection and maintain systemic homeostasis and diminish side effects from virus exposure. Leveraging therapies currently being used to improve gut health could be exploited for better disease outcomes during severe disease. Additional studies on the gut-brain axis, particularly from diverse populations across the world, are warranted.

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REVIEW

Hepatitis B virus infection modeling using multi-cellular organoids derived from human induced pluripotent stem cells

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Abstract

Chronic infection with hepatitis B virus (HBV) remains a global health concern despite the availability of vaccines. To date, the development of effective treatments has been severely hampered by the lack of reliable, reproducible, and scalable *in vitro* modeling systems that precisely recapitulate the virus life cycle and represent virus-host interactions. With the progressive understanding of liver organogenesis mechanisms, the development of human induced pluripotent stem cell (iPSC)-derived hepatic sources and stromal cellular compositions provides novel strategies for personalized modeling and treatment of liver disease. Further, advancements in three-dimensional culture of self-organized liver-like organoids considerably promote *in vitro* modeling of intact human liver tissue, in terms of both hepatic function and other physiological characteristics. Combined with our experiences in the investigation of HBV infections using liver organoids, we have summarized the advances in modeling reported thus far and discussed the limitations and ongoing challenges in the application of liver organoids, particularly those with multi-cellular components derived from human iPSCs. This review provides general guidelines for establishing clinical-grade iPSC-derived multi-cellular organoids in modeling personalized hepatitis virus infection and other liver diseases, as well as drug testing and transplantation therapy.

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Core Tip: The development of an effective treatment for hepatitis B virus relies on reliable and reproducible *in vitro* modeling systems. Recently, three-dimensional multicellular organoid systems have attracted considerable attention owing to their superior susceptibility and capability to precisely recapitulate the virus life cycle. Recent advances in organoid-generating strategies, particularly those derived from human induced pluripotent stem cells, together with future improvements in genetic modification and scalability, will undoubtedly promote personalized disease modeling and drug development.

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INTRODUCTION

Hepatitis B virus (HBV), a highly prevalent global health concern, is among the most common causes of advanced chronic liver disease development. Globally, 292 million people (approximately one-third patients in China) reportedly present with chronic HBV infection[1], which is associated with a wide range of clinical manifestations, including liver cirrhosis, liver failure, and hepatocellular carcinoma[2]. Despite the availability of approved vaccines, currently, HBV treatment is mainly restricted to interferon (IFN) and nucleoside analogs, which rarely achieve absolute hepatitis elimination[3]. Notably, clinical treatment usually results in different responses and outcomes due to viral genotype diversity and patient genetic susceptibility, which further increases treatment difficulties[4]. To date, *in vitro* culture systems have been utilized to determine the characteristics and life cycle of hepatitis C virus. Furthermore, these systems have been proven to substantially contribute to our understanding of the genomic replication subsystems that ultimately led to productive viral infection, as well as successful identification of small molecules having effective activity against various hepatitis C viral enzymes[5]. Therefore, to better understand the HBV life cycle and to promote drug development, a robust personalized *in vitro* HBV modeling platform is urgently needed to recapitulate the entire HBV replication cycle, especially that of covalently closed circular DNA (cccDNA) infection and formation. Several *in vitro* modeling systems have been developed, mostly based on *in vitro* culture systems using primary hepatocytes or hepatic cells from other sources[6-9]. Besides, sodium taurocholate cotransporting polypeptide (NTCP)-transformed hepatocellular carcinoma cell lines are also available. No one system fits all studies, but findings from different systems may be complementary. Maintenance of hepatic function and increase of susceptibility to HBV infection *in vitro* are the principal focus for most established systems. In this regard, numerous culture systems have also been developed to better fit the in *vivo* microenvironment.

Considering the demand for clinical applications, the reproducibility and scalability of modeling systems have generated interest in recent years. Accordingly, as a theoretically unlimited source of stem cells without ethical implications, pluripotent stem cells [induced pluripotent stem cell (iPSC)] represent the most promising source that can be used to yield substantial quantities of homogeneous and reproducible cellular components of the liver. Moreover, with a patient-specific genetic background, iPSC-derived hepatocyte-like cells (HLCs) are optimal for individualized disease modeling, which may remarkably benefit mechanistic studies and drug development for hereditary diseases and diseases closely related to the host genetic background, such as HBV infection[10-12]. With considerable expression of major mature hepatocyte markers, as well as host factors required for HBV infection, including the NTCP, iPSC-

HLCs could support robust production of HBV particles and viral RNAs[7,13]. More importantly, significant inhibition of HBV infection was detected upon anti-HBV agent treatment, suggesting that iPSC-HLCs could be utilized as a novel HBV infection model for drug testing[14].

However, the drawbacks in maturation and difficulty in maintenance of iPSC-HLCs have raised considerable concerns. Liver organoids (LOs) can be maintained for a longer time, are more susceptible to HBV infection, and exhibit enhanced liver function[3]. Multi-cellular three-dimensional (3D) organoid culture systems have recently become an effective strategy to compensate the deficiencies and also extend the potential modeling duration. With supportive mesenchymal and endothelial cells in the 3D microenvironment, HBV susceptibility was greatly enhanced compared to monolayer culture system, while the duration for HBV propagation and virus production was also prolonged[15]. HBV-infected LOs could serve as a reliable and viable *ex vivo* infection model for hepatitis research to investigate the role of host genetic background in HBV infection and individual prognosis of infection, enabling personalized hepatitis treatment[15]. To further enhance the reproducibility and scalability, an all-iPSC-based strategy has been adopted most recently in generating multi-cellular LOs with improved hepatic functions almost equivalent to those of adult hepatocytes, although their advantages in HBV modeling remains to be confirmed in future studies[16]. With the development of 3D expansion strategies, together with advances in direct programming or induction of liver fate, novel engineered LOs are expected to be established on a large scale for broad applications in disease modeling, drug screening, and transplantation.

Here, we summarized the recent advances in *in vitro* modeling systems for HBV infection, especially using multi-cellular LOs. Additionally, we discussed the current and future challenges in the application of advanced organoid generation platforms in terms of efficiency, reproducibility, and scalability. Finally, we explored the future applications of multi-cellular LOs in personalized and precise treatments.

ADVANCES AND LIMITATIONS IN MODELING HEPATITIS B INFECTION

Cell sources

HBV only efficiently infects fully differentiated hepatic cells. Until now, primary human hepatocytes (PHHs) were the only permissive agents used for the study of HBV infection *in vitro*[17]. This system remains the gold standard and is utilized to model HBV infections *in vitro*[18]. However, fresh PHHs have markedly limited availability and unpredictable variability among donors. Furthermore, PHHs have extremely limited replication ability in conventional culture systems and cannot be easily subjected to genetic manipulation, which hinders scaled-up manufacturing and targeted gene therapy. Moreover, PHHs show rapid loss of their mature hepatic functions and infection susceptibility after plating. Thus, these cells are unsuitable for utilization as a stable source for long-term modeling and testing[19]. For a reliable and practical platform for long-term disease modeling and drug development, a new gold standard should be established.

To overcome restrictions in the use of PHHs, especially source availability and cost, researchers have shifted their attention to immortalized tumor-derived or transformed hepatocytes and hepatocellular carcinoma cell lines, such as Huh7, HepG2, HepG-2.2.15, HepAD38, HepaRG, HepDE19, HepBHAe82, and HepG2-NTCP cells[3,20,21]. HepG2.2.15 cells can stably express HBV viral gene products. However, HBV viral particles are derived from chromosomally integrated DNA rather than cccDNA during *in vivo* processing. Later, a highly HBV-permissive cell clone of HepAD38 cells was created, which could produce more robust viruses. However, this cell line failed to recapitulate the complete viral replication process[20,21]. In this regard, the liver progenitor cell line HepaRG is a more attractive source for modeling, since these cells show morphological and functional features similar to those of primary hepatocytes. More importantly, HepaRG cells are susceptible to HBV infection upon supplementation with corticoids and DMSO, which maintains the cellular maturation state [6]. However, this infection system has a relatively low infection efficiency, even under the strict culture conditions necessary to prevent dedifferentiation. Additionally, cccDNA amplification did not occur in this system[7]. Subsequently, to meet the requirements for high-throughput screening of cccDNA-targeting drugs, a HepDE19 cell line was developed in which the expression of HBV e antigen (HBeAg) was derived from cccDNA. However, the ELISA antigen shows cross-reactivity with viral HBeAg homologues, which dramatically diminishes the assay specificity[22,23]. To

address this problem, the HepBHAe82 cccDNA reporter cell line was developed based on similar principles. Importantly, this line produced high levels of cccDNA-dependent HBeAg with high, specific readout signals and did not disrupt any cis-elements that were essential for HBV replication and HBeAg secretion[21]. As a potential tool for cccDNA-targeted drug screening and testing, it remains to be determined whether cccDNA formation, stability, and transcription are recapitulated in this system. To further achieve high susceptibility to HBV infection, NTCP, an essential receptor for HBV infection[24] was introduced into liver cancer cell lines. Establishment of susceptible cell lines, such as HepG2-NTCP, permitted highly efficient HBV infection and enabled the identification of key events or processes in the viral life cycle, although this system could not be used to elucidate the entire HBV life cycle[25]. Furthermore, the physiological and functional characteristics of tumor-derived or transformed hepatoma cell lines are distinct from those of normal hepatocytes. Comparing the HepG2 transcriptome to cells obtained from liver tissue biopsies showed that most genes with upregulated expression in HepG2 cells were associated with carcinogenesis, while those with upregulated expression in the liver were enriched in heterologous biometabolism[26], thereby implying a fundamental deficiency of hepatocellular carcinoma cell lines for reliable and precise modeling. Besides, a non-hepatic cell line was recently developed for HBV modeling. Constructed by exogenous expression of human NTCP, HNF4α, RXRα, and PPARα, the 293T cell line (293T-NE-3NR) was able to support HBV entry, transcription, and replication, although the HBV production (HBV DNA, cccDNA, and pgRNA) in 293T-NE-3NR remained lower than that in the HepG2 cell line. To date, the use of a non-hepatic model is quite a new concept; however, it may serve as a beneficial complement to the current hepatocellular carcinoma cell models. Without intrinsic liver-related host factors, an exogenous construction strategy may help discover the key factors involved in HBV infection[27].

To achieve functional hepatocytes that closely resemble primary hepatocytes, HLCs differentiated from pluripotent stem cells have attracted considerable attention as a novel cell source. Since the use of embryonic stem cells is markedly hindered due to ethical issues, iPSCs induced by the ectopic expression of defined transcription factors (*e.g.*, *Oct4*, *Sox2*, *Klf4*, and *c-Myc*) in patient fibroblasts or other somatic cells have emerged as the most promising source for generating HLCs[28-30]. After conduction of stepwise hepatic differentiation[31-33], iPSC-derived HLCs could support effective HBV infection and last for a long time[14,34]. Together with their infinite expansion capability, iPSCs have enabled the provision of numerous hepatic cell sources necessary for stable and scalable modeling with less variability than PHHs[25]. Meanwhile, the epigenetic effects in human iPSCs are minimal and the genetic differences between individual donors contribute to the major heterogeneity between different iPSC lines[35]. In this regard, iPSCs may serve as stable, reliable, and powerful platforms for the precise analysis of the hepatitis virus in specific genotypes and for modeling infection in individuals with different genetic backgrounds[29,36,37]. Additionally, induced hepatocytes (iHeps) generated by direct reprogramming from somatic cells share similar hepatic features and may be potentially used to support productive HBV infection[34].

Despite the progressive improvements reported thus far, *in vitro* hepatocyte induction with transcription factors, growth factors, or chemicals cannot fully represent the complete phenotypic spectrum of PHHs. Further, inhibition of the innate immune response remains an obstacle to achieve efficient infection[38]. Thus, improvements including optimized induction strategies and niche signaling are warranted to generate transcriptional/functional and hepatitis virus-friendly hepatic sources to recapitulate *in vivo* virus-host recognition.

In vitro microenvironment

The biological characteristics and functions of primary hepatocytes depend considerably on the *in situ* liver microenvironment[39,40]. Simply covering hepatocytes with multiple layers of collagen gel can markedly extend the culture time and help retain cellular functions[41]. Nevertheless, utilization of the conventional *in vitro* culture systems results in a rapid loss of hepatocyte polarity and associated liver function[42], innate immune responses, and susceptibility to HBV infection due to the unsatisfied intrinsic requirements of the liver microenvironment. Consequently, most of these HBV infection systems require the addition of DMSO, PEG[43], or immunomodulatory agents to achieve essential susceptibility, which is not feasible for practical considerations[34]. *In vivo*, however, HBV can infect more efficiently[44], which highlights that the native hepatic microenvironment, including the physiological liver architecture and niche signaling, markedly impacts productive HBV infection.

As the most abundant non-parenchymal hepatic cell population, liver sinusoidal endothelial cells (LSECs) can efficiently enhance HBV infection by secreting epidermal growth factor (EGF)[45] and can lead to the development of liver fibrosis by constitutively expressing the major histocompatibility complex I-restricted antigens and co-stimulatory molecules, which shifts the immune balance of the liver toward tolerance[46]. Additionally, hepatic stellate cells (HSCs) maintain hepatocyte function *via* cell signaling and secretion of the necessary factors[47]. Furthermore, HSCs are actively involved in the development of hepatitis virus-induced hepatic fibrosis[48]. Kupffer cells (KCs) and Ly6C+ monocytes are closely associated with host defense in the liver and play crucial roles in the development of anti-HBV immunity. Particularly, KCs are involved in hepatitis virus recognition, suppression of infection, immune cell recruitment and activation, and are correlated with HBV progression and outcomes [49,50]. The integration of multi-cellular interactions as a whole requires precise imitation of the native liver niche. Conventionally, monolayer co-culture systems are used as a simple tool to recapitulate cell-cell interactions and signaling. However, these systems provide non-natural physiological conditions, in which only parts of the hepatocyte surface establish communication with the adjacent cells or the extracellular matrix (ECM), leaving the rest of the cell exposed to the culture medium. Although certain aspects of hepatitis or other viral infections may be elucidated using this system, there remain major concerns regarding a physiologically intact host for virus recognition and replication. Cells assembled in 3D aggregates are more similar to cells *in vivo*[15]. PHHs cultured in 3D systems preserve certain metabolic functions and permit more accurate hepatotoxicity prediction during *in vitro* modeling[42]. However, the liver microenvironment includes the 3D mass of hepatocytes and an organized architecture consisting of numerous cell types, which synergistically regulate liver-specific physiology. Therefore, reconstruction of these interactions will theoretically help in the development of a more reliable system for the initiation and long-term maintenance of the hepatitis infection cycle. Recent achievements in generating multi-cellular organoids have paved the way for the establishment of an accurate model for obtaining fundamental knowledge on disease progression, including HBV infection [15], particularly when single patient-derived iPSCs contribute to the cellular composition of desired organoids[51]. iPSC-derived multi-cellular LOs maintain mature hepatic phenotypes and functions to levels comparable to those of PHHs. More importantly, LOs are more susceptible to HBV infection than iPSC-HLCs and maintain long-term HBV propagation while producing infectious viruses. Together with the genetic characteristics inherited from the donor iPSCs, LOs help in the achievement of a promising individualized infection model[15].

In static culture systems, gas exchange, nutrient supply, and waste removal remain significant challenges. The emergence of microfluidic technology has enabled the control of the culture system with optimized temperature, pH, nutrients, and gas exchange, while providing microscale structures and parameters that may help to obtain an approximate simulation of the *in vivo* microenvironment[52,53]. Thus, the application of these systems permits organoid maintenance in *in vivo*-like physiological states or even in disease-like conditions in a controlled manner. Notably, improved drug sensitivity can be obtained using microfluidic 3D systems compared to static culture systems. With the utilization of further engineering approaches to control the initial cellular composition, shape, and size of cell aggregates; cell-cell and cell-ECM[54] interactions; and biochemical gradients similar to *in vivo* microenvironment, the incoming generation of organoids-on-a-chip has considerable potential for large-scale applications in high-throughput drug testing and screening.

Micropatterning

Highly structured organs and tissues provide a fundamental *in vivo* microenvironment for cells, in which cell polarity and functions are consistently influenced by the specific boundaries imposed by the neighboring cells and the ECM. The mechanical and spatial properties of the microenvironment are tightly correlated with intracellular signaling pathways and affect cell transcriptome status and function. In the past decade, numerous studies have been conducted and have provided insights into the geometrical modeling of the *in vitro* culture microenvironment by developing engineered substrates that precisely mimic the composition, structure, and mechanical properties of a specific organ or tissue.

To date, accumulative *in vitro* studies show that cell differentiation can be directed by using micropatterned substrates. The micropattern features on the spreading area and the aspect ratio determine the differentiation status of human mesenchymal stem cells (MSCs)[55]. Moreover, hydroxyapatite bioceramic-based hybrid structures stimulate osteogenic differentiation of MSCs by activating integrins, the BMP2

signaling pathway, and cell-cell communication. The width of micropattern stripes regulates vascular smooth muscle cell orientation by regulating cell elongation[56]. Similarly, mechanical and spatial control was observed during pluripotent stem cell differentiation. When these cells are differentiated within geometrically uniform and circular micropatterns, the patterning of regionalized cell fate can be reproducibly recapitulated[57,58]. Moreover, recent evidence shows that a decreased patterning width promotes iPSC differentiation efficiency, suggesting that stricter topographies direct cell fate specification[59]. Interestingly, the micropattern stiffness, degradability, and biochemical composition can even promote pluripotency reprogramming, thus emphasizing the critical role of micropatterns in morphogenetic and functional remodeling[60].

Although micropatterning strategies are increasingly applied in cell-based modeling and testing, they pose challenges in ensuring compatibility with rapidly advancing organoid technology that requires the use of non-adhesive substrates and extensive cell-cell interactions for self-organization. To this end, scaffold-dependent micropatterning strategies were adopted in recent studies conducted on distinct epithelial organoid generation. Meanwhile, structural and functional improvements have been increasingly reported. For example, a microfilament-based floating scaffold was developed to perform micropatterning and to guide brain organoid self-organization with increased reproducibility and improved tissue architecture[61]. Additionally, a spatially confined hydrogel scaffold was developed to guide self-organization of tube-shaped functional intestinal organoids with an accessible lumen and a physiologically relevant spatial arrangement of crypt- and villus-like domains[62]. Similarly, in terms of LOs, both colloidal crystal and hydrogel-based scaffolds were found to support advanced liver functions[63,64]. However, to date, the mechanism of spatial microenvironmental control of organ/tissue-specific cell fate specification remains undetermined. Once identified, scaffold-based micropatterning systems are expected to offer a potential platform for generating liver-specific and regionalized cell types simultaneously from a single iPSC. These platforms will be of remarkable importance for constructing multi-cellular organoids with native spatial composition, and will help to elucidate the mechanisms by which intra- or intercellular signals regulate distinct cellular identities and maturation patterns, and will fundamentally mimic the *in vivo* physiological features and hepatic functions for precise hepatitis modeling.

CURRENT CHALLENGES IN GENERATING MULTI-CELLULAR LIVER ORGANOIDS

Liver-like cellular organoid composition and reconstitution

To better mimic an *in vivo* niche resembling liver-specific function and physiology for disease modeling, vascularized functional multi-cellular LOs were first developed in 2013[65]. However, in this study, human MSCs and umbilical vein endothelial cells (HUVECs) were used to substitute the liver intrinsic stromal cellular components, including HSCs and LSECs, which remain major obstacles for future clinical applications. To address this problem, a complete all-iPSC-based strategy was established, in which hepatic functions were promoted by using iPSC-septum transversum mesenchymal and iPSC-endothelial progenitor cells. In both global transcriptome and function, the new system was more advanced than the HUVEC/MSC-based strategy and was comparable to primary adult hepatocytes[16]. However, this system remains deficient in recapitulating the immune response and inflammation due to the lack of an essential liver component, KCs. Recently, KCs, hepatocytes, stellate cells, and biliary cells were successfully induced synchronously in a multi-cellular system by facilitating co-differentiation from iPSCs, which represents a real and more complex liver-like tissue system that may be used for modeling inflammatory and fibrotic responses[51]. With advanced integration and optimization of cellular composition (Table 1), multi-cellular LOs are expected to support precise and more complex disease modeling.

With the progressive understanding of liver organogenesis and technical advancements in iPSC differentiation methods toward tissue-specific cellular compositions, the following aspects remain as future challenges: (1) The procedures that can be used to remodel the intrinsic cellular composition proportion and distribution in LOs; (2) Their specific roles in liver development and disease processes; (3) Clarification of biochemical signatures of liver-specific ECM; and (4) The use of clinically compatible ECM hydrogels or microparticles. Overcoming these challenges will be beneficial for maintaining LO physiological characteristics during modeling.

Novel strategies for synchronous induction

Before the all-iPSC-based strategy was adopted, the development of multi-cellular organoids included the collection and separate preparation of each cell type and subsequent self-organization into a 3D structure. Although the emergence of all-iPSC-based strategies facilitated the establishment of a reproducible and stable source for clinical and pharmaceutical applications, the procedure for diverse cell co-culture remains time-consuming, labor-intensive, inefficient, and cannot meet the requirements for large-scale production and practical applications. Moreover, selection of the specific culture medium and ECM to simultaneously maintain multiple cell lineages remains challenging.

To address these limitations, a stepwise organoid induction and maintenance platform was developed, which involved sequential stimulation with the addition of growth factor cocktails into the culture medium. Following the induction of foregut spheroids by retinoic acid (RA) treatment and subsequent hepatic maturation, iPSCs were simultaneously differentiated into multi-cellular LOs. However, this approach lacks precise control over multi-lineage specification and physiological functions. Furthermore, the optimization of medium and growth factors that are used to direct and maintain distinct cell specificity remains an obstacle. In this regard, systematic probing of the molecular pathways and transcriptional networks has emerged as a more precise method for direct organogenesis *in vitro*. *PROX1* and *ATF5* overexpression combined with *CYP3A4* activation enables gene regulatory network (GRN)-based engineering and facilitates direct programming or induction and the development of iPSC-derived multi-cellular LOs. Importantly, compared to primary mature hepatocytes, the established GRNs not only showed major similarities in natural liver functions, but were also responsive to perturbation and feedback regulation, and thus were superior to immature HLCs and fetal LOs[66]. Although this new method enabled the development of multi-cellular systems *via* synthetic genetic control, it is difficult to reflect every facet of human liver physiology and function, such as relatively low levels of urea production and CYP2C19 activity. Further global assessment of GRNs may be necessary to identify critical regulatory signatures for advanced liver fate. Additionally, it is important to carefully determine and avoid potential tumorigenesis by transcription factor-based genetic programming and delocalization processes using guide RNAs (gRNAs) to achieve clinical safety standards.

Taken together, synchronous induction or programming systems may substantially improve the efficiency of traditional multi-lineage differentiation, which is a promising strategy for practical applications (Figure 1). However, the lack of maturation, purity, and batch-to-batch variability remains a major challenge. Further optimization of stepwise induction or programming strategies, including medium, supplements, timing, and application of tissue scaffolding and microfluidic devices, is warranted to precisely recapitulate the intrinsic multi-cellular organ system *in vivo*.

Scaling up

The possibility of scaled-up applications is a major advantage of using *in vitro* organoid systems over animal models. The increasing biomedical and preclinical demands for high-throughput disease modeling, drug testing, and screening, have led to the identification of critical bottleneck of scaling up, which prevents their application to provide more reliable, rapid, and cost-effective modeling.

Using chemical induction, a limited expansion capability of human hepatocytes was successfully induced in 2D monolayer culture systems[67,68]. However, expansion in a 3D system is subjected to complicated factors, such as nutrient/gas exchange, contact inhibition, and complex cell-cell signaling. Although long-term expansion of organoids from human adult biliary epithelial-derived progenitor cells, fetal/adult hepatocytes, and pluripotent stem cell-derived hepatocytes has been successfully established, the expansion efficiency remains markedly lower than that observed with the application of 2D systems[69-73] (Table 2). Recently, a rotating flask-based method was developed for the large-scale expansion of human LGR5-positive liver stem cell organoids. In this system, organoids were subjected to continuous passage and stable maintenance for at least 6 wk, which was mostly achieved with improved oxygenation[74].

Scaling up multi-cellular systems is another major challenge. By developing an omni-well array culture platform, Takebe *et al*[16] adopted an all-iPSC strategy for the large-scale production of homogenized and vascularized LOs in a clinical setting and achieved a 10^8-cell grade, which seemed to be feasible for human therapeutic applications. However, this platform involved the performance of labor-intensive work, including separate preparation of diverse cell sources for co-culture, thus limiting the efficacy of scaling. Later, the same group established a synchronous co-differentiation

Table 1 Current modeling strategies and applications of human multi-cellular liver organoids

Model/application	Multiple cells (ratio)	Culture system	Advances/significance	Limitations	Ref.
ALF	iPSC-HEs, HUVECs, BM-MSCs (10:8:2)	3D, Matrigel	Multi-cellular LOs with vascularization	Low reproducibility; Time-consuming	2013[65]
ALF	HEs, MCs, ECs (all from iPSCs) (10:8:2)	3D, ULA	All-iPSC-based strategy	Time-consuming; High cost	2017[16]
ALF	iPSC endoderm cells, HUVECs, UC-MSCs (10:7:1)	3D, ULA	LOs generated from single donor-derived cells	Low reproducibility; Time-consuming	2018[75]
ALF	iPSCs, HAMECs (3:1)	EB, Agarose	HAMECs improved hepatic functions	Unable to reflect the nature cellular composition of liver	2019[102]
Liver fibrosis	HepaRG, THP-1, hTERT-HSC	3D, Hanging drop	LOs derived from cell lines	Functional deficiency	2017[103]
Liver fibrosis and steatohepatitis	PHHs, KCs, HSCs, SECs (16:2:1:1)	3D, ULA	LOs derived from primary cell sources	Low reproducibility; High cost	2018[104]
Steatohepatitis	Hepatocytes, HSCs, BCs, KCs (all from iPSC)	3D, ULA	Co-differentiation of multiple cell lineages for iPSC- LOs	Functions undetermined; Potential inter/intra-batch variability	2019[51]
HBV infection *ex vivo*	iPSC endoderm cells, HUVECs, BM- MSCs (10:7:1)	3D, ULA	Validation of advantages of iPSC-LOs in HBV modeling	Low reproducibility; Time-consuming	2018[15]
Hepatic differentiation	iPSC-HEs, MSCs, HUVECs (10:2:7)	3D, Matrigel	Platform to identify developmental paracrine signals involved in hepatocyte differentiation	Low reproducibility; Time-consuming	2017[105]
Hepatic differentiation	iHEPs, ECs, HSCs, cholangiocytes (10:7:2:1)	3D, ULA	Cholangiocytes impaired the hepatic functions in LOs and were associated with the liver disease relevant phenotype	Cholangiocyte activation in LOs was unclear	2019[106]
Liver development and angiogenesis	Hepatocytes, BCs, ECs, HSCs (all from iPSC)	3D, Matrigel	Engineered iPSC-LOs by programming of the gene regulatory network	Not completed for liver functions	2021[66]

ALF: Acute liver failure; BCs: Biliary cells; BM: Bone marrow; EB: Embryoid body; ECs: Endothelial cells; HAMECs: Human adipose microvascular endothelial cells; HBV: Hepatitis B virus; HEs: Hepatic endoderm cells; HSCs: Hepatic stellate cells; HUVECs: Human umbilical vein endothelial cells; iHEPs: Induced hepatocytes; KCs: Kupffer cells; Los: Liver organoids; MCs: Mesenchymal cells; MSCs: Mesenchymal stem cells; NPCs: Non-parenchymal cells; PHHs: Primary human hepatocytes; SECs: Sinusoidal endothelial cells; UC: Umbilical cord; ULA: Ultralow adhesion microwell plate.

method that facilitated the generation of multi-lineage organoids in a preset growth factor administration strategy, thereby saving substantial time and costs for co-culture procedures[51]. Most recently, organoid programing has emerged as a promising strategy for establishing both functionality and scalability. A tissue-specific set of factors were identified using computational analyses. These factors were then genetically introduced into LOs, and after manipulation of GRNs, multi-lineage tissue fate could be directed to develop multi-cellular systems. Notably, these LOs can be passaged and cryopreserved while retaining their vascular networks[66]. This scalable organoid production system may pave the way for personalized disease modeling, drug testing, and even transplantation. However, there remain challenges in the identification of liver-specific GRNs to directly re-establish the entire profile of human liver physiology and function. Other tissue engineering techniques, such as pinning bioreactors, microfluidics, and bioprinting may be developed before high-throughput and highly efficient platforms can be established to meet the practical demands of pharmaceutical and clinical applications.

Transplantation and gene editing

In addition to demonstrating utility as an *in vitro* tool in the modeling of a structural and functional organ unit, organoids are also potential transplantation substitutes for organ donors. Indeed, organoids exhibit various advantages over conventional cell-based strategies, as mentioned elsewhere[69,75]. By mimicking liver regeneration after injury, an advanced *in vitro* system was recently developed for the long-term expansion of functional LOs. Particularly, LOs generated from primary liver sources have demonstrated genetic stability after long-term culture[69,70], suggesting that these

Table 2 Up-to-date methods for obtaining expandable human liver organoids

Initial cells	Expansion systems (substrate for embedding)	Medium	Expansion capability (Split ratio/passage days/expansion duration)	Ref.
Fetal and adult hepatocytes	3D, Matrigel	AdDMEM/F12, B27, N-Acetylcysteine, gastrin, RSPO1, Noggin, Wnt, EGF, FGF7, FGF10, HGF, TGFa, Nicotinamide, A83-01, CHIR99021 and Y27632	1:3/7-8 d/> 16 passages	2018[70]
$EpCAM^+$ bile duct cells	3D, Matrigel or BME gel	AdDMEM/F12, N2, B27, N-Acetylcysteine, gastrin, RSPO1, Noggin (d0-3), Wnt (d0-3), EGF, FGF10, FGF19, HGF, Nicotinamide, A83-01, FSK, and Y27632 (d0-3)	1:4-1:8/7-10 d/6 mo	2015[69]
$EpCAM^+$ bile duct cells	3D, BME gel	AdDMEM/F12, B27, N-Acetylcysteine, gastrin, RSPO1, Noggin (d0-3), Wnt (d0-3), EGF, FGF10, FGF19, HGF, Nicotinamide, A83-01, FSK, and Y27632 (d0-3)	1:5/7-10 d/> 6 mo	2019 [107]
iPSC derived $EpCAM^+$ hepatic progenitors	3D, Matrigel	AdDMEM/F12, B27, N-Acetylcysteine, gastrin, RSPO1, Noggin (d0-3), Wnt (d0-3), EGF, FGF10, HGF, Nicotinamide, A83-01, FSK and Y27632 (d0-3)	1:4-1:8/7-10 d/9-12 mo	2019[71]
PSC-derived hepatocytes	3D, Matrigel	AdDMEM/F12, N2, B27, N-Acetylcysteine, gastrin, RSPO1, EGF, FGF10, HGF, Nicotinamide, A83-01, FSK	1:3-1:10/7 d/3 mo	2019[72]
PSCs transduced with *PROX1*, *ATF5* and *CYP3A4*	3D, Matrigel	APEL medium	NA/10 d/17 d	2021[66]

APEL: A commercial medium from Stem Cell Technologies; BME: Basement membrane extract; bFGF: Basic fibroblast growth factor; BME: Basement membrane extract; EGF: Epidermal growth factor; FGF: Fibroblast growth factor; FSK: Forskolin; HGF: Hepatocyte growth factor; iPSCs: Induced pluripotent stem cells; PSCs: Including embryonic stem cells and induced pluripotent stem cells; RSPO1: R-spodin1.

organoids are a safe transplantation source for clinical applications. Alternatively, autologous transplantation of iPSC-derived LOs is expected to be promising for the realization of reproducible and personalized liver disease treatment. As a highly prevalent disease, HBV infection remains a strong risk factor for developing liver cirrhosis, hepatocellular carcinoma, or other end-stage liver diseases, thus posing a major threat to health worldwide[76]. To this end, there exists a necessity of developing effective treatments for chronic HBV infection. Currently, the standard therapy for chronic hepatitis B infection includes two major agents. First, nucleotide or nucleoside analogs such as tenofovir and entecavir interfere with viral replication by suppressing the synthesis of reverse transcriptase; second, IFNs such as IFNα induce long-lasting immunological control[76]. However, available drugs show side effects or high response variability, and drug resistance leads to reduced long-term effectiveness. Currently, HBV infection cannot be absolutely cured[77]. Combined with the CRISPR/Cas9 genome editing technology, patient iPSC-derived organoid transplantation may provide a new therapeutic strategy for realizing a highly specific HBV treatment. As the functional regions in key HBV receptors (*e.g.*, NTCP) for HBV binding and post-binding entry have been identified, specific genetic knock-out or modification may markedly change HBV infection susceptibility, thereby permitting the development of transplanted organoids that are resistant to HBV infection. More importantly, the risk of HBV recurrence may be permanently avoided, which remains a major concern after liver donor transplantation and is associated with graft dysfunction or failure and cirrhosis progression[78].

Nevertheless, current research on organoids is in the preclinical stages, and clinical safety and efficacy remain to be established for adult or iPSC-derived LO transplantation. Moreover, the potential risks associated with genetic modification, tumorigenesis, and transplantation techniques should be emphasized in light of individual and societal values. In the context of continuing investigation regarding clinical concerns[79,80], quality-controlled and personalized LOs from authorized patient-derived cell banks are expected to be used routinely in the future.

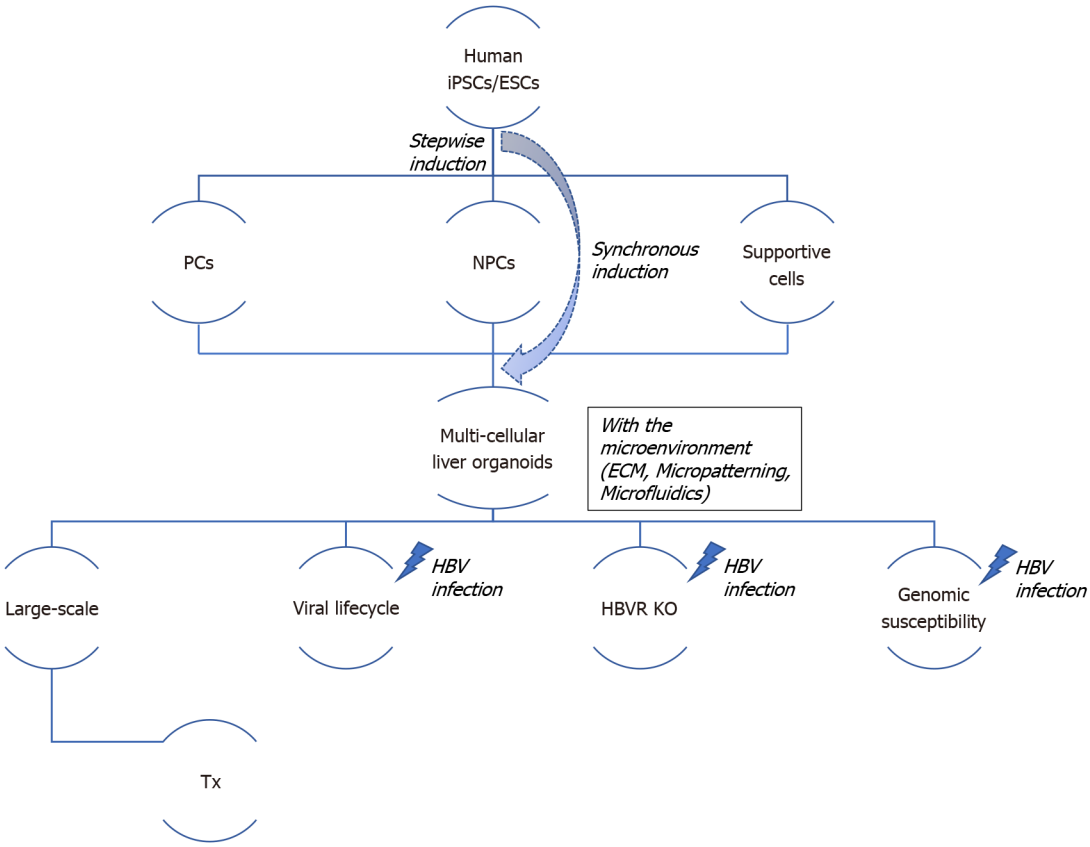


Figure 1 Schema to generate human liver organoids and strategy regarding their application potential and hepatocyte B virus modeling. Multi-cellular liver organoids are generated and self-organized with parenchyma cells, non-parenchyma cells, and other hematopoietic or/and neuronal supportive cells after stepwise induction from human induced pluripotent stem cells/embryonic stem cells; in contrast, synchronous induction using a CRISPR-based strategy markedly enhances their hepatic functions and improves their reproducibility and scalability. The personalized and population organoid system may provide a reliable platform for high-throughput hepatocyte B virus (HBV) drug screening, allowing to understand novel key points of the HBV lifecycle, gene editing to knock-out the HBV receptor, and distinguish genomic susceptibility in a large population. Transplantable multi-cellular organoids without the HBV receptor have much potential for future applications. ECM: Extracellular matrix; ESCs: Embryonic stem cells; HBV: Hepatocyte B virus; HBVR: Hepatocyte B virus receptor; iPSCs: Induced pluripotent stem cells; KO: Knock-out; NPCs: Non-parenchyma cells, including Kupffer cells, hepatic stellate cells, and liver sinusoidal endothelial cells; Supportive cells: Such as neuronal cells, hematopoietic cells, and vascular endothelial cells; PCs: Parenchyma cells such as hepatocytes; Tx: Transplantation.

PERSPECTIVES

Automated and large-scale cultural systems

Robust, consistent, and cost-effective manufacturing is extremely important for facilitating the practical applications of LOs for high-throughput disease modeling, drug testing, and therapeutic purposes. Similarly, automatic LO generation and culture systems may provide promising opportunities to meet critical requirements, especially in reproducibility and scalability.

In recent years, automated culture systems have garnered considerable attention, particularly in the maintenance and differentiation of iPSCs. Since the quality of iPSCs is dependent on the technical skills applied during culture and manipulation[81], automated culture systems can decrease technical variability. With the development of fully automated cell culture systems that have revolutionized cell seeding, medium changing, imaging, harvesting, and analysis, human iPSCs can be maintained in an undifferentiated state for a long period[82]. Additionally, reprogramming and differentiation of cardiomyocytes, hepatocytes, mesenchymal cells, neural stem cells, and retinal pigment epithelial cells have also been successfully performed using automated systems, with high quality and consistency[83-86]. Recently, an automated system was first used to produce LOs. Although the differentiation process was not fully automated, automatic LO self-assembly and maintenance substantially permitted the obtainment of reliable and reproducible results, suggesting potential applications in applied research and industry[87]. With the development of differentiation strategies and medium optimization for iPSC-derived parenchymal and non-parenchymal liver cells, the integration of automated cell production and maintenance systems is expected to further promote a completely standardized high-throughput workflow for

LO production. These workflows will enable downstream applications such as HBV and liver cancer modeling, drug screening, and regenerative medicine.

Genetic manipulation

Genetic manipulation seems extremely promising in the revelation of the biological functions of specific genes, their regulation networks, and their relevance to disease progression[88]. Moreover, genetic manipulation facilitates drug testing in various disease states with different genetic backgrounds. In recent years, the CRISPR/Cas9 technology has markedly simplified genetic engineering because of its versatility and broad application potential. Combined with the development of organoid generation and culture technology, CRISPR has enabled the establishment of multiple disease models using genetically engineered tissue-specific organoids. For example, colorectal cancer models were derived from intestinal organoids with mutations in *APC*, *SMAD4*, *TP53*, and *KRAS*[89]; injury models were derived from kidney organoids *via* podocalyxin knock-out[90]; brain tumor models were derived from cerebral organoids with mutations in *MYC* and mutations commonly found in glioblastoma[91]; and liver cancer models including hepatocellular carcinomas and intrahepatic cholangiocarcinomas were derived from LOs with mutations in *c-Myc* or *RAS*[92]. Notably, genetically modified iPSC-derived organoids may permit broader practical applications in modeling genetic diseases once consistent patient-derived organoids are developed, such as those with A1AT deficiency or Allagile syndrome. Although not reported, iPSC-derived LOs are expected to acquire altered susceptibility to HBV infection with mutated NTCP expression, which is critical in studying viral replication.

In addition to demonstrating versatility as an *in vitro* tool used for the elucidation of substantial clues to diseases, gene correction in organoid models provides a potential strategy for future gene therapy. Using the CRISPR/Cas9 genome editing system, the mutation of cystic fibrosis transmembrane conductor receptor was corrected in intestinal organoids derived from cystic fibrosis patients, which restored normal function[93]. Subsequently, mutation correction was achieved in retinal organoids derived from retinitis pigmentosa patients using a similar strategy[94]. With increasing interest in the genetic manipulation of various tissue-specific organoids, the development of a robust genome editing strategy is necessary to further enhance organoid-based disease modeling and related gene correction. Recently, CRISPR–Cas9-mediated homology-independent organoid transgenesis (CRISPR-HOT) was developed to efficiently generate knock-in human organoids[95], thus providing a powerful platform for obtaining reliable and scalable applications in this field.

However, to date, no gene editing has been performed on multi-cellular organoids, partially because of the technical difficulties in targeting specific cell types within the system. Additionally, there are several issues that are encountered with gene therapy, including off-target mutations, delivery difficulties, and the lack of standardized tests to assess anti-HBV gene therapy[96,97]. Undoubtedly, new innovations in the rapidly evolving field of the CRISPR technology may provide exciting possibilities for organoid-based clinical applications.

Susceptibility to HBV infection

The identification of NTCP, the specific receptor for HBV[98], paved the way to construct NTCP-expressing hepatoma cell lines that were originally not susceptible to HBV. Exogenous NTCP expression may render these cell lines vulnerable to HBV infection, providing a more reliable system for studying the HBV life cycle. However, this system does not help elucidate the entire HBV life cycle and permits limited viral spread, with only moderate amounts of detectable cccDNA, partially due to intrinsic deficiencies of these cell lines. As a result, an extremely high multiplicity of infection is necessary to achieve substantial infection, and the addition of polyethylene glycol is needed to enhance infection in most reported cases[99]. Meanwhile, hepatoma cells are physiologically impaired in several intracellular signaling pathways and functions after forced NTBC expression, which limits their use for studying virus-host interactions[38].

In addition to NTCP, HBV infection may also depend on other receptors, such as the sialic acid glycoprotein receptor (ASGPR)[100,101]. A comparison of iPSC-derived hepatic progenitors and HLCs revealed a significant increase in ASGPR expression in iPSC-HLCs, suggesting that ASGPR might be considered a candidate receptor for hepatitis virus infection in mature hepatic cells and might mediate HBV entry into cells. To date, NTCP expression is considered the most important factor for HBV infection in host cells[13]. However, it remains necessary to identify other unknown receptors or media that contribute to hepatitis virus infection susceptibility. The emergence of 3D multi-cellular LOs, which mimic the native liver microenvironment,

has attracted substantial attention owing to its superior HBV susceptibility compared to 2D culture[15]. Although the underlying mechanism remains unclear, these organoids may provide an opportunity for the investigation of potential receptors and related pathways involved in viral infection. Computational analyses, followed by sequential knock-out of the candidate targets in engineered multi-cellular LOs, may enable the complete understanding of the key genes contributing to HBV susceptibility. Furthermore, the role of ethnic genotypes in viral susceptibility may be studied using this system. Individuals of different races present with different susceptibilities to hepatitis virus. For example, Chinese populations have a high susceptibility to hepatitis B, while European and American populations are prone to hepatitis C infection. It would be beneficial to use iPSC-derived LOs from different genomic backgrounds to compare HBV infection in different populations with the same risk factors, or to compare susceptibility in the same population with different risk factors. In this regard, drugs, which may account for disparities in different populations and individuals, may also be developed.

CONCLUSION

With the progressive understanding of liver organogenesis, the development of 3D organoid culture systems has helped in the establishment of a novel platform for precise and personalized liver disease modeling. However, it remains particularly challenging to promote the efficiency, reproducibility, and scalability of organoid reconstitution and maintenance. It is expected that future advances in genetical engineering and automated culture system will put 3D multi-cellular organoids into a variety of practical uses, including hepatitis infection modeling and related drug development. Furthermore, the proof of concept for clinical-grade patient iPSC-derived multi-cellular LOs is expected and will contribute much to modeling and treatment of hepatitis virus infection and other liver diseases.

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MINIREVIEWS

Artificial intelligence in colonoscopy

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Abstract

Colorectal cancer remains a leading cause of morbidity and mortality in the United States. Advances in artificial intelligence (AI), specifically computer aided detection and computer-aided diagnosis offer promising methods of increasing adenoma detection rates with the goal of removing more pre-cancerous polyps. Conversely, these methods also may allow for smaller non-cancerous lesions to be diagnosed *in vivo* and left in place, decreasing the risks that come with unnecessary polypectomies. This review will provide an overview of current advances in the use of AI in colonoscopy to aid in polyp detection and characterization as well as areas of developing research.

Key Words: Colonoscopy; Artificial intelligence; Computer-aided detection; Detection; Characterization; Computer-aided diagnosis

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Core Tip: The rapidly evolving field of artificial intelligence (AI) has found many applications in the field of colonoscopy. Specifically, we describe the technologies that have been developed to detect and characterize colonic polyps with the goal of real-time analysis as well as minimizing the risks of avoidable polypectomies. Additionally, we discuss some of the future directions of AI in this area including advancements in robotic technology.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of cancer related death in men and women in the United States. The incidence of CRC has been declining for over 30 years due in part to screening colonoscopies that detect and remove pre-cancerous polyps[1].

The adenoma detection rate (ADR) is a metric used by endoscopists representing the percentage of time at least one adenoma is detected on screening colonoscopies[2]. Adenoma detection rates differ widely among endoscopists, between 7% to 52%, with higher ADRs associated with a decreased risk of CRC. It is now recommended that endoscopists target an ADR target ≥ 25%[3]. Artificial intelligence (AI), specifically computer-aided detection (CADe) software is being studied to detect polyps during colonoscopy with the goal of increasing adenoma detection rates[2,4,5].

Broadly, artificial intelligence (AI) relates to the ability of a computer program to obtain outside data (*e.g.*, images) and to subsequently take independent actions towards a particular goal (*e.g.*, pattern identification). Machine learning is a form of AI that relies on the analysis of large datasets in order to make predictions that can be used for decision making. Deep learning, a subtype of machine learning, uses an artificial neural network comprised of layers of interconnected "computing units" that mimic biological neural connections and allow for complex "understanding" of input data. This neural network allows the computer program to learn independently from unstructured input data. Many times a deep learning program can process a large number of photos, independently identify patterns among them, and then use that information to make predictions about new images. This powerful technology that has been used to train machines in image and sound recognition is now being applied in the medical field in the form of computer-aided diagnosis and detection, which applies AI and computer vision technologies to the diagnosis of various pathologies. The technology is rapidly expanding in areas like colonoscopy where there is significant room to mitigate human error in visual diagnosis.

Many polyps detected and resected during colonoscopies are diminutive polyps (≤ 5 mm), and a significant number of these are non-neoplastic. Polypectomy increases the risk of complications during colonoscopy, including the risk for bleeding and perforation[6]. Computer-aided diagnosis (CAD) technology has been studied to characterize the histology of polyps *in vivo*.

The American Society for Gastrointestinal Endoscopy's Preservation and Incorporation of Valuable Endoscopic Innovations (PIVI) initiative has provided guidelines that aim to reduce the cost and need for pathological assessments in addition to reducing the risks associated with polypectomy. The PIVI's first guideline is known as "resect and discard", which entails resecting the diminutive polyp and discarding if the CAD technology used to characterize the polyp has a similar surveillance interval compared to the traditional pathology assessment (≥ 90%). The PIVI's second guideline allows the endoscopist to leave hyperplastic diminutive polyps in place in the rectosigmoid area if the CAD technology has a NPV ≥ 90% for characterizing adenoma histology[7].

COLORECTAL POLYP DETECTION

The first study to use CADe to detect colorectal polyps was published in 2003[8-11]. Karkanis *et al*[8] used wavelet transformation technology to detect polyps with a sensitivity of 93.6% and specificity of 99.3%. Years later, deep learning networks were applied to CADe, which has paved the way for *in vivo* and real-time analysis studies.

Urban *et al*[2] was the first to use CADe for polyp detection in real-time. The study assessed 9 standard colonoscopy videos. At the time of the colonoscopies, 28 polyps were identified and removed. Using CADe, an additional 17 polyps were detected, compared to only an additional 8 that were identified by an expert endoscopist[2,9]. Klare *et al*[12] then applied CADe in real time and *in vivo* to 55 colonoscopies. The ADR of the CADe was similar to the endoscopists' (29.1% and 30.9%, respectively). However, the CADe was inferior in detecting flat and small polyps.

More recently, there have been randomized control trials (RCTs) performed in real-time using colorectal polyp detection technology. Wang *et al*[4] included 1058 patients in a non-blinded study, 536 were randomized to colonoscopy and 522 were randomized to colonoscopy with CADe. The ADR was statistically superior in the colonoscopy with the CADe group compared to the control group (29.1% *vs* 20.3%). Moreover, CADe was better at detecting diminutive adenomas, but there was no difference in detection rate for polyps larger than 5 mm. Notably, the CADe did not miss any polyps, but had 39 false positives alarms for polyps[5]. Wang *et al*[4] then performed a double blinded RCT in which patients were randomized to a colonoscopy with sham system (n = 478) or a colonoscopy with CADe (n = 484) group. Results showed the ADR was 34% in the CADe group, which was superior to the control group, 28%. Gong *et al*[13] randomized 704 patients in a partially blinded RCT to CADe assisted colonoscopy or control standard colonoscopy. Similarly, the ADR was significantly better in the CADe group than the control group (16% compared to 8%, respectively). Repici *et al*[14] performed a similar nonblinded RCT and found an ADR of 54.8% in the colonoscopy with CADe group, which was significantly better than the ADR for the control group (40.4%). The CADe was also able to detect more adenomas that were < 10 mm in size compared to the control group. The authors also found that there was no significant difference in withdrawal time (excluding biopsy time) of the endoscope between the two groups. Liu *et al*[15] randomized 1026 patients to CADe or control groups. The ADR was significantly better in the CADe group (39%) than the control group (23%). The CADe did not miss any polyps and there were only 36 false positive alarms. It was also noted that the withdrawal times between groups were similar (CADe 6.16 minutes compared to control 6.11 min).

Su *et al*[16] created an automatic quality control system (AQCS) to improve aspects of colonoscopy using a deep learning model. They randomized 659 patients and found that the AQCS group had a superior ADR than the control group (28.9% *vs* 16.5%). However, they found the AQCS had a longer withdrawal time (excluding biopsy time) compared to the control group (7.03 ± 1.01 min *vs* 5.68 ± 1.26 min).

Recent meta-analyses have concluded that CADe was accurate at detecting adenomas[17,18]. Barua *et al*[17] included 5 RCTs (with a total of 4311 patients) and concluded the ADR was significantly better using CADe with colonoscopy (29.6%) than colonoscopy alone (19.3%), with a false positive alarm mean of 11.2%. Lui *et al*[18] analyzed 6 studies that used CADe and found the accuracy of CADe was 90% with sensitivity and specificity of 95% and 88% respectively. In both studies, colonoscopy with CADe improved detection of diminutive adenomas[17,18].

A significant limitation of CADe technology is the potential to have high false positive alarm rates. Even though Wang *et al*[5] and Liu *et al*[15] had low rates, Hassan *et al*[19] reported a total of 1092 false positive alarms, which averaged 27.3 per colonoscopy. Also, many of the studies assessing CADe had control groups with low ADRs between 8%-23%[5,13,15,16], which is below the recommended target ADR of ≥ 25%[3].

Table 1 summarizes the key recent studies in colorectal polyp detection.

POLYP CHARACTERIZATION

Going beyond merely identifying polyps, AI has been applied to provide real-time *in vivo* diagnoses of neoplastic *vs* non-neoplastic lesions. A number of different modalities for this have been described over the years.

White light endoscopy

Traditional white light (WL) endoscopy is the most familiar modality used by endoscopists today. High-definition white light (HDWL) endoscopy is the most recent improvement in this area. Recent randomized controlled trials have demonstrated that HDWL is non-inferior to other modalities, specifically narrow band imaging (NBI) and chromoendoscopy[20-22]. In 2016, Rex *et al*[20] found that there was no statistically significant difference in the number of serrated lesions detected in 804 patients randomized to undergo either WL or NBI endoscopy. The same year, Klare *et al*[21] published the results of a trial that randomized 380 patients to either HDWL or NBI. They also found no statistically significant advantage of one modality over the other in distinguishing between neoplastic and non-neoplastic polyps. Yang *et al*[22] randomized 210 patients with ulcerative colitis to undergo colon cancer screening with HDWL or chromoendoscopy and found no significant difference in dysplasia detection rates between the two modalities (5.6% for HDWL *vs* 3.9% for chromoen-

Table 1 Colorectal polyp detection

Ref.	Study design	Algorithm type	Dataset	Results
Karkanis *et al*[8]	Retrospective	CADe (Wavelet Decomposition)	180 images	Sensitivity: 93.6% Specificity: 99.3%
Urban *et al*[2]	Retrospective	CADe (DCNN)	8461 images &20 colonoscopy videos	Accuracy: 96.4% False Positive: 7%
Klare *et al*[12]	Prospective*In vivo*	CADe	55 colonoscopies	ADR of: CAD 29.1% and Endoscopist 30.9%
Wang *et al*[5]	Non-blinded RCT	CADe using Shanghai Wision AI Co. Ltd. (DCNN)	Randomized 522 patients to CADe and 536 to control group	ADR of CAD 29.1% *vs* control 20.3%
Wang *et al*[4]	Double blinded RCT	CADe using EndoScreener (DCNN)	Randomized 484 patients to CAD and 478 to sham system	ADR of CAD 34% *vs* control 28%
Gong *et al*[13]	Partially blinded RCT	CADe using ENDOANGEL (DCNN)	Randomized 355 patients to CAD and 349 to control	ADR of CAD 16% *vs* control 8%
Repici *et al*[14]	Partially-blinded RCT	CADe using GI-Genius (CNN)	Randomized 341 patients to CAD and 344 to control	ADR of CAD 54.8% *vs* control 40.4%
Liu *et al*[15]	Non-blinded RCT	CADe using Henan Xuanweitang Medical Information Technology Co. Ltd (convolutional 3D network)	Randomized 508 patients to CAD and 518 control	ADR of CAD 39% *vs* control 23%
Su *et al*[16]	Partially blinded RCT	Automatic quality control system (ACQS)(DCNN)	Randomized 308 patients to AQCS and 315 to control	ADR of AQCS 28.9% *vs* control 16.5%

CADe: Computer-aided detection; CAD: Computer-aided diagnosis; DCNN: Deep convolutional neural network; ADR: Adenoma detection rate.

doscopy).

In 2017, Komeda *et al*[23] designed and tested a CAD system based on a convolutional neural network to augment WL endoscopy. It functioned as an AI system that trained with previously collected colonoscopy images to assist endoscopists in detecting and diagnosing colon polyps during WL endoscopy. After training on 1200 images, their CAD-neural network system correctly differentiated between adenomatous and non-adenomatous polyps in 70% of newly presented cases[23].

This strategy of applying AI to the detection and classification of colorectal lesions using WL endoscopy has continued to be an area of active study. Researchers have shown that CAD, using both convolutional neural networks and deep learning models, promises to identify suspicious lesions and accurately classify them[24-26]. Zheng *et al*[24] developed a convoluted neural network (CNN) to be used with WL endoscopy. They trained their AI system with over 600 polyp-containing images from independent public databases and found that their diagnostic model had a sensitivity of 68.3% and a precision of 79.3% when applied to 196 new polyp-containing images [24]. However, they found significant variation in model performance depending on which image database was used to train the CNN, and they also only trained and tested their CNN on still images[24]. Going beyond polyp identification, Yang *et al*[26] developed a deep learning (DL) model to assist in classification of colorectal lesions during WL endoscopy. They trained their model on 3828 images and validated it on a set of 240 new images. When classifying lesions as neoplastic *vs* non-neoplastic, their model had a sensitivity of 95.4% and specificity of 30.1%[26]. Their model was also able to classify advanced lesions (high grade dysplasia and stages T1-T4 CRC *vs* non-advanced (tubular adenomas and non-neoplastic lesions) with a sensitivity of 80.0% and specificity of 91.3%[26].

A recent prospective crossover study conducted by Wang *et al*[25] compared traditional WL colonoscopy to CAD-assisted colonoscopy in 369 patients. Patients requiring colonoscopy underwent either traditional WL colonoscopy or CAD-assisted colonoscopy immediately followed by the other, such that each study participant underwent both methods. They found that the adenoma miss rate was 40% for those undergoing traditional colonoscopy first *vs* 14% in those undergoing CAD-assisted colonoscopy first[25]. Polyp detection followed a similar trend with a miss rate of 46% in those undergoing traditional colonoscopy first *vs* 13% in those undergoing CAD-assisted colonoscopy first[25]. Interestingly, of the adenomas missed, participants undergoing CAD-assisted colonoscopy were less likely to have polyps under 5mm and under 10 mm missed, suggesting that CAD is particularly helpful in identifying

smaller lesions[25].

The key studies for polyp characterization using white light endoscopy are summarized in Table 2.

Narrow band imaging

Narrow band imaging (NBI) is used to enhance visualization of vascular patterns in the epithelium of lesions to aid in the classification of polyps[27-29]. However, training and experience is needed to operate NBI; therefore, studies have applied computer-aided diagnosis to NBI[27,29-34].

Tischendorf *et al*[29] was the first to apply CAD to magnified NBI. The CAD evaluated 209 polyps and assessed for three vessel features on each NBI image, then a support vector machine was used to classify the polyp as neoplastic or non-neoplastic. It had an accuracy of 85.3%, sensitivity of 90% and specificity of 70.2%. When compared to the consensus of the investigators (accuracy 91.9%, sensitivity 93.8%, and specificity 85.7%), the CAD was inferior. Gross *et al*[27] performed a similar study with 434 polyps, but assessed for nine vessel features. The CAD had an accuracy of 93.1%, sensitivity of 95%, specificity of 90.3%, and NPV of 92.4%, which was comparable to the results of the expert endoscopists and superior to the novice endoscopists.

Years later, Byrne *et al*[30] and Chen *et al*[31] improved CAD by creating deep learning models to analyze NBI images and categorize polyp histology. Both studies only included diminutive polyps (≤ 5 mm). Chen *et al*[31]'s deep learning model assessed still NBI images of 284 polyps and classified polyps with an accuracy of 90.1%, sensitivity of 96.3%, specificity of 78.1%, PPV of 89.6%, and NPV of 91.5%. The authors also studied the diagnosis time, which was statistically faster for the deep learning model than both expert and novice investigators[31]. Byrne *et al*[30]'s deep learning model assessed 125 endoscopy NBI video segments of polyps. Of the classified polyps, the accuracy was 94% with sensitivity 98%, specificity 83%, PPV 90%, and NPV 97%. However, the model was not able to classify 15% of polyps due to a lack of confidence. All of the CAD with NBI studies named above with the exception of Tischendorf's initial study met the PIVI criteria for resect and discard or diagnose and leave in situ[7,27,30,31].

Few prospective studies have been performed with CAD using NBI imaging to classify polyp histology. Kominami *et al*[32] evaluated 118 polyps with NBI. The CAD had an accuracy of 94.9%, sensitivity of 95.9%, specificity of 93.3%, PPV of 95.9%, and NPV 93.3%. The authors used this data to investigate colonoscopy surveillance interval which did not change in 38 of the 41 patients when using the CAD results to classify polyps. Mori *et al*[33] also performed a study assessing CAD when used with NBI on 466 diminutive polyps. The NPV for rectosigmoid neoplastic polyps ranged from 95.2% to 96.5% depending on the worst or best case scenario respectively.

Most recently, Song *et al*[35] created a CAD using a deep learning model and tested it *in vivo* by sending still NBI images during the colonoscopy to a computer. The CAD then categorized the histology in real time. The polyps were classified as serrated, benign adenoma, or deep submucosal cancer with an accuracy of 82.4%, which was superior to trainees (63.8%), but inferior to expert endoscopists (87.3%). The accuracy of trainee endoscopists improved with the addition of CAD to 82.7% showing that CAD can increase the accuracy in this group.

Table 3 provides a summary of the key recent studies of polyp characterization using narrow band imaging.

Laser-induced fluorescence spectroscopy

Another strategy currently under investigation to optically diagnose lesions during endoscopy is laser-induced fluorescence spectroscopy. This diagnostic method relies on low-power laser radiation to induce fluorescence in tissues that can differentiate normal from neoplastic lesions[36]. In recent years, CAD systems have been developed to analyze the fluorescent spectra produced when tissues are exposed to a laser. These CAD systems take advantage of the differences between the fluorescence of normal and pathological tissue to predict the likelihood that a lesion is abnormal.

Kuiper *et al*[37] and Rath *et al*[38] studied a laser-induced fluorescence spectroscopy system designed to be used in real-time to help clinicians make decisions regarding biopsy and resection of concerning lesions. However, it is important to note that the accuracy of the algorithm used by Kuiper *et al*[37] was 73.4% and the NPV only 74.4%, falling short of the performance thresholds of the American Society for Gastrointestinal Endoscopy's PIVI initiative for diminutive lesions. The pilot study conducted by Rath *et al*[38] was more promising, with an overall accuracy of 84.7%, sensitivity of 81.8%, specificity of 85.2%, and NPV of 96.1%. A 2017 randomized controlled trial by Min *et al*[39] was further able to demonstrate that use of linked color

Table 2 White light endoscopy

Ref.	Study design	Algorithm type	Dataset	Results
Komeda *et al*[23]	Diagnostic model development	CAD-neural network combination to assist WL endoscopy	1200 training images then tested on 10 new images	Cross-validation accuracy: 0.751
Zheng *et al* [24]	Diagnostic model development	WL endoscopy using YOLO (CNN)	196 WL images from an independent public database	Accuracy: 79.3% Sensitivity: 68.3%
Wang *et al* [25]	Prospective crossover study	Traditional WL endoscopy *vs* CAD colonoscopy	369 patients from a single hospital in China	Adenoma miss rate of 13.9% in the CAD group *vs* 40% in the traditional group, $P < 0.0001$
Yang *et al* [26]	Diagnostic model development	Validation of a deep learning model called "ResNet-152" to classify colorectal lesions	3828 WL colonoscopy images from 1339 patients	Mean model accuracy: 79.2% for advanced CRC, early CRC/HGD, TA, and non-neoplastic AUC: 0.818

CAD: Computer-aided diagnosis; WL: White light; CNN: Convoluted neural network.

imaging technology (which enhances the colors produced by laser endoscopic modalities) improved overall polyp detection rate when compared to traditional white light endoscopy (polyp detection rate 73% for WL and 91% with linked color imaging).

The recent key studies of laser-induced fluorescence spectroscopy are summarized in Table 4.

Autofluorescence endoscopy

Autofluorescence imaging (AFI) is a form of image enhanced endoscopy that differentiates tissues based on their various abilities to capture and reflect fluorescent light [40]. Similar to laser-induced fluorescence spectroscopy, this method takes advantage of endogenous reflective properties of various tissues (fluorophores), but instead of using a laser emitting an exact wavelength of light, AFI uses incoherent light sources. This technology aims to visually highlight tumors, which have more heterogeneous fluorescence on their surface compared to normal colonic mucosa. This image-enhancement displays normally fluorescing mucosa as green and abnormally fluorescing mucosa as red/purple.

In a 2019 study of 802 patients randomized to undergo either AFI endoscopy or white light endoscopy, Takeuchi *et al*[41] found that using AFI during endoscopy increased the number of flat neoplasms detected overall compared to WL, especially in the ascending colon. However, the overall detection rate of advanced neoplasms was not significantly improved with AF compared to WL[41]. In a meta-analysis of 11 studies, Wanders *et al*[42] calculated that the sensitivity and specificity of autofluorescence imaging for the optical diagnosis of colonic lesions were 86.7% and 65.9% respectively. This led the authors to conclude that AFI is not as reliable as other methods for visual diagnosis of colonic neoplasms. This was further confirmed in a 2018 meta-analysis by Imperatore *et al*[43] which found no significant difference between the dysplasia detection rates between AFI and WL (OR = 1.42, 95%CI: 0.74-4.11) when combining the results of two randomized controlled trials representing 92 patients undergoing surveillance colonoscopy.

More recently, researchers have developed CAD systems that can further characterize the images obtained during AFI endoscopy using software that can calculate the green to red light ratios of various tissues encountered during colonoscopy[44-47]. Such developments may help differentiate lesions from normal mucosa in cases where the green to red variation is less obvious, improving on the results of the studies looking at the use of AFI without the use of CAD.

Arita *et al*[44] created a color-contrast index (CCI) for AFI. Their CCI was developed from 54 colorectal lesions found in 43 patients who underwent either WL or AFI endoscopy. They found that as the CCI increased (*i.e.*, greater contrast between the lesion and the adjacent normal tissue), so did the malignant potential of the assessed lesion (*i.e.*, carcinomas had higher CCIs on average compared to adenomas)[44]. Aihara *et al*[45] expanded on this idea by using color-analysis software to calculate red/green ratios (RGR) for 102 Lesions in 32 patients undergoing AFI endoscopy. In their study, they were able to differentiate neoplastic from non-neoplastic lesions with sensitivity of 94.2%, specificity of 88.9%, PPV of 95.6%, and NPV of 85.2%[45]. In a similar study, Inomata *et al*[46] also calculated RGRs to distinguish between non-

Table 3 Narrow band imaging

Ref.	Study design	Algorithm type	Dataset	Results
Tischendorf *et al*[29]	Prospective *Ex vivo*	CAD – NBI (support vector machine)	209 polyp images	Accuracy: 85.3% Sensitivity: 90% Specificity: 70.2%
Gross *et al*[27]	Prospective *Ex vivo*	CAD – NBI (support vector machine)	434 polyp images	Accuracy: 93.1% Sensitivity: 95% Specificity: 90.3% NPV: 92.4%
Chen *et al*[31]	Retrospective	CAD – NBI (DCNN)	284 polyp images	Accuracy: 90.1% Sensitivity: 96.3% Specificity: 78.1% PPV: 89.6% NPV: 91.5%
Byrne *et al*[30]	Retrospective	CAD—NBI (DCNN)	125 polyp videos	Accuracy: 94% Sensitivity: 98% Specificity: 83% PPV: 90% NPV: 97%
Kominami *et al*[32]	Prospective	CAD –NBI (support vector machine)	118 polyps	Accuracy: 94.9% Sensitivity: 95.9% Specificity: 93.3% PPV: 95.9% NPV: 93.3%
Mori *et al*[33]	Prospective	CAD – NBI (support vector machine)	466 polyps	NPV: 95.2% to 96.5%
Song *et al*[35]	Prospective *In vivo*	CAD –NBI (DCNN)	363 polyps	Accuracy: 82.4%

CAD: Computer-aided diagnosis; NBI: Narrow band imaging; DCNN: Deep convolutional neural network.

neoplastic lesions, adenomas plus superficial cancers, and deep cancers. They were able to characterize hyperplastic polyps and neoplastic lesions, with sensitivity of 83.9%, specificity of 82.6%, PPV of 53.1%, and NPV of 95.6%[46]. Additionally, they were able to differentiate between adenomas plus superficial cancers and deep submucosal cancers with a sensitivity of 80.0%, specificity of 84.4%, PPV of 29.6%, and NPV of 98.1%[46].

In 2019, Horiuchi *et al*[47] developed software to calculate real-time RGR ratios with the specific goal of identifying diminutive neoplastic rectosigmoid polyps (≤ 5 mm). Using their CAD-assisted AFI endoscopy, they identified 429 diminutive polyps (258 rectosigmoid) in 95 patients. The endoscopists then confirmed whether the lesions identified with the CAD-assisted AFI were actually diminutive neoplastic polyps with trimodal imaging endoscopy (TME) combining findings of WL, AFI, and NBI. The CAD-assisted AFI software was able to identify diminutive neoplastic polyps with a sensitivity of 80.0%, specificity of 95.3%, PPV of 85.2%, and NPV of 93.4%[47].

Table 5 contains a summary of the key recent studies involving autofluorescence endoscopy.

Magnifying chromoendoscopy

In the technique of magnifying chromoendoscopy, suspected colonic lesions are washed with proteinases and colored with an indigo carmine or crystal violet solution in order to allow better visualization of the surface under magnification up to 150 times[48]. In a systematic review, Brown *et al*[49] showed that chromoendoscopy

Table 4 Laser-induced fluorescence spectroscopy

Ref.	Study design	Algorithm type	Dataset	Results
Kuiper *et al*[37]	Diagnostic model development	Diagnostic performance of WavSTAT	87 patients	Accuracy: 73.4% NPV: 74.4%
Rath *et al* [38]	Diagnostic model development	Diagnostic performance of WavSTAT for predicting polyp histology	27 patients	Accuracy: 84.7% Sensitivity: 81.8% Specificity: 85.2% NPV: 96.1%
Min *et al* [39]	Randomized controlled trial	Linked color imaging with laser endoscopic system *vs* WL	141 patients from 3 hospitals in China	Polyp detection rate of 91% in the LCI group, 73% in the WL group, $P < 0.0001$

WL: White light.

Table 5 Autofluorescence endoscopy

Ref.	Study design	Algorithm type	Dataset	Results
Arita *et al*[44]	Diagnostic model development	Calculation of a color-contrast index (CCI) for AFI	43 patients who underwent both WL and AF endoscopy	Sensitivity: 95.3% Specificity: 63.6%
Aihara *et al*[45]	Diagnostic model development	CAD-assisted AF	32 patients undergoing colonoscopy in a Japanese hospital	Sensitivity: 94.2% Specificity: 88.9% PPV: 95.6% NPV: 85.2%
Inomata *et al* [46]	Diagnostic model development	CAD-assisted AF	88 patients	Accuracy: 82.8% Sensitivity: 83.9% Specificity: 82.6% PPV: 53.1% NPV: 95.6%
Horiuchi *et al* [47]	Diagnostic model development	CAD-assisted AF	95 patients undergoing colonoscopy	Accuracy: 91.5% Sensitivity: 80.0% Specificity: 95.3% PPV: 85.2% NPV: 93.4%

AFI: Autofluorescence imaging; WL: White light; CAD: Computer-aided diagnosis; AF: Autofluorescence.

significantly increased both the number of patients with polyps (OR = 1.87, 95%CI: 1.51-2.3) and neoplasms detected (OR = 1.53, 95%CI: 1.31-1.79). Kudo *et al*[48] demonstrated that certain pit patterns on magnifying chromoendoscopy are associated with malignancy, showing sensitivity of 97.8%, specificity of 91.4%, and accuracy of 97.1%. Kanao *et al*[50] demonstrated that magnifying chromoendoscopy can be used to differentiate severely irregular lesions from those with only mild irregularities, a key distinction as 56.1% of the former are associated with deep submucosal invasive

adenomas *vs* only 6.7% of the latter.

Several automated computer-based systems have been developed for analysis of pit patterns. The system developed by Takemura *et al*[51] was able to accurately diagnose 132 out of 134 (98.5%) of images captured with magnifying chromoendoscopy demonstrating that artificial intelligence aided systems can reliably predict histological changes compared to endoscopists. Häfner *et al*[52] used texture analysis of magnified chromoendoscopic images to achieve accuracy as high as 99.59%. Recent work by Qi *et al*[53] further showed that artificial intelligence can be used to quantify colonic crypts and provide objective measures of area, density, eccentricity, solidity, straightness, and parallelism which in turn can be used to reduce variability compared to human observation.

Recent studies on the use of magnifying chromoendoscopy in polyp characterization are summarized in Table 6.

Endocytoscopy

The technique of endocytoscopy uses a contact light microscope attached to a colonoscope to provide endoscopic images with ultra-magnification up to 520 times. The addition of staining allows for real-time histological diagnoses to be made. Studies have shown that the accuracy of this technique is comparable to traditional biopsy [54]. However, a major limitation is the need for expert experience in order to make real-time diagnoses. CAD has been developed in response to this shortcoming.

In 2015, Mori *et al*[54] described the use of an endocytoscopic imaging computer-aided diagnostic system. In this study, 39 non-neoplastic and 176 neoplastic small colorectal polyps less than 10 mm in size were analyzed by artificial intelligence software and compared to the results of both expert and trainee endoscopists. They showed comparable sensitivity (92% *vs* 92.7%) and accuracy (89.2% *vs* 92.3%) of the computer-aided diagnostic system compared to experts[54]. Moreover, the artificial intelligence program performed significantly better than trainee endoscopists who only had sensitivity of 81.8% and accuracy of 80.4%[54]. Takeda *et al*[55] described the development of a CAD system that used 5543 endocytoscopic images for machine learning. Following this, 188 images of a mix of adenomas and invasive cancers were analyzed by the CAD system and compared to pathological diagnoses with 89.4% sensitivity, 98.9% specificity, 98.8% accuracy, 98.8% PPV, and 90.1% NPV[55]. Moreover, the system used a support vector machine to calculate the probability of results being classified accurately. The study specifically looked at high-confidence diagnoses described as those having a ≥ 90 % probability of being correct. Out of 188 images analyzed, 134 fell into this category with 98.1% sensitivity, 100% specificity, 99.3% accuracy, 100% PPV, and 98.8% NPV[55].

In 2018, Mori *et al*[33] showed the efficacy of real-time endocytoscopy with CAD in detecting diminutive polyps ≤ 5 mm in size. 466 polyps were assessed with 98.1% pathologic prediction rate, 93.8% sensitivity, 90.3% specificity, and 94.1% PPV. Moreover, they were able to demonstrate overall negative predictive value of 96.4% which is significantly above the threshold for a "diagnose-and-leave" treatment strategy. However, the study only demonstrated 65.8% NPV for lesions proximal to the recto-sigmoid area[33].

In 2020, Kudo *et al*[56] performed a study to evaluate the efficacy of an AI system that uses endocytoscopic images to look at cell nuclei, crypt structure, and microvessels. It was able to identify malignant lesions with 96.9% sensitivity, 100% specificity, 98% accuracy, 100% PPV, and 94.6% NPV. Compared to expert endoscopists with 92.8% sensitivity, 94.3% specificity, and 93.9% accuracy as well as trainee endoscopists with 70.8% sensitivity, 65.7% specificity, and 69% accuracy, this CAD system significantly outperformed both groups[56].

Table 7 summarizes the key recent studies in endocytoscopy.

Confocal endomicroscopy

Anatomic variation can hinder accurate traditional endoscopic biopsy. Confocal endomicroscopy produces high resolution magnification of the mucosal layer of the gastrointestinal tract using laser illumination with simultaneous detection of light reflected from the tissue through a narrow pinhole. By filtering out light that is scattered from angles outside of layers corresponding to mucosa in question, this technique allows for high spatial resolution and real-time endoscopic evaluation of targeted areas of tissue. Compared to normal colonic mucosa with well-organized crypt structures, malignancy causes irregularities and interruptions[57].

André *et al*[58] designed software that used probe-based confocal laser endomicroscopy to automatically classify colonic polyps. 135 images with both neoplastic and nonneoplastic polyps were analyzed by the automated software with 92.5% sensitivity,

Table 6 Magnifying chromoendoscopy

Ref.	Study design	Algorithm type	Dataset	Results
Takemura *et al*[51]	Partially blinded retrospective study	CAD using HuPAS	134 pit pattern images	Accuracy: 98.5%
Häfner *et al*[52]	Partially blinded retrospective study	CAD using Dual-Tree Complex Wavelet Transform	484 RGB pit pattern images	Accuracy: 99.59%
Qi *et al*[53]	Diagnostic model development	CAD using automated imaged analysis	79 colon samples (14 normal, 44 normal tissue adjacent to cancer, 21 malignant)	Automated segmentation achieved precision ratio of 0.69 and match ratio of 0.73

CAD: Computer-aided diagnosis.

83.3% specificity, and 89.6% accuracy which was not significantly different compared to diagnosis by two expert endoscopists with 91.4% sensitivity, 85.7% specificity, and 89.6% accuracy. Ştefănescu *et al*[59] retrospectively analyzed 1035 endomicroscopic images processing them through a CAD system that allowed for feature identification *via* fractal analysis of glandular structures showing that homogeneity and feature number were significantly different in malignancy. In turn, this was used to design an artificial intelligence program with a diagnosis error rate of 15.5%[59].

One problem with early probe-based confocal endomicroscopic images is the need for high-level magnification which leads to a long learning-time for automated image interpretation. Taunk *et al*[60] showed the efficacy of a CAD algorithm that utilized a lower magnification method with a wider field-of-view necessitating less images. The algorithm demonstrated similar sensitivity, specificity, and accuracy compared to expert endoscopists (95% *vs* 98%, 94% *vs* 95%, and 94% *vs* 96% respectively) and significantly better performance than less experienced endoscopists who only had sensitivity of 60%, specificity of 85%, and accuracy of 73%[60].

The recent key studies on confocal endomicroscopy are summarized in Table 8.

FUTURE DIRECTIONS

Automated polyp detection and characterization

Many studies have been performed using AI to detect or classify the histology of colorectal polyps; however, little research has been done on simultaneous detection and classification of colorectal polyps using AI. Mori *et al*[33] combined previously studied CADe and CAD systems to develop AI technology that is able to detect then characterize the colorectal polyps[61,62]. White-light imaging was used to detect polyps with an accuracy of 94%[61,62]. Then, classification of the polyps was then performed using magnified NBI with a NPV of 95.2%[33,61]. Ozawa *et al*[34] created a deep convolutional neural network (DCNN) that detected polyps using white light or NBI with a sensitivity of 92% and a PPV of 86%. The DCNN then classified the polyps using either white light with an accuracy of 83% and NPV of 90% or NBI with an accuracy of 81% and NPV of 91%. Further research is needed in concurrent automated detection and characterization of colorectal polyps.

Robotics

Much of the work in robotics has centered around the use of self-propelling colonoscopes and less on polyp detection. Recent studies on the use of robotics in colonoscopy are summarized in Table 9.

Eickhoff *et al*[63] demonstrated the first use of a novel computer-assisted colonoscope in 2007 that would change shape at 16 different segments depending on insertion depth in an effort to decrease discomfort from colonoscope looping. While it required an endoscopist to steer the scope, the device used CAD to change shape as it was advanced. The device was able to intubate the cecum in 100% of patients with no complications at discharge, 48 h, and 30 d[63].

In 2016, Pullens *et al*[64] demonstrated the utility of colonoscopy with robotic steering and automated lumen centralization (RS-ALC). In a study of 18 endoscopists including 8 experts and 10 novices, the addition of RS-ALC significantly improved the time to intubate the cecum in novices (8 min 56 s compared to baseline 11 min 47 s without RS-ALC) as well as polyp detection rate (88.1% *vs* 78.6%)[64]. However,

Table 7 Endocytoscopy

Ref.	Study design	Algorithm type	Dataset	Results
Mori *et al* [54]	Pilot study	CAD using EC-CAD	176 colorectal polyps from 152 patients	Accuracy: 89.2% Sensitivity: 92% Specificity 79.5%
Takeda *et al* [55]	Retrospective study	CAD using EC-CAD	5543 endocytoscopy images for machine learning. 200 test images	Overall Accuracy: 94% Sensitivity: 89.4% Specificity: 98.9% PPV: 98.8% NPV: 90.1% High-confidence diagnosis Accuracy: 99.3% Sensitivity: 98.1% Specificity: 100% PPV: 100% NPV: 98.8%
Mori *et al* [33]	Single-group, open-label, prospective study	Real-time CAD during colonoscopy	466 diminutive polyps from 325 patients	Accuracy: 98.1% Sensitivity 93.8% Specificity 90.3% PPV 94.1% NPV 89.8%
Kudo *et al* [56]	Retrospective study	CAD using EndoBRAIN	100 polyps from 89 patients	Accuracy: 98% Sensitivity 96.9% Specificity 100% PPV 100% NPV 94.6%

CAD: Computer-aided diagnosis.

similar results were not seen with expert endoscopists whose time to intubate the cecum actually increased (13 min 1 s compared to baseline 2 min 9 s without RS-ALC) and polyp detection rate decreased (69% *vs* 80.9%)[64]. Slawinski *et al*[65] demonstrated the use of an *in vivo* autonomously controlled highly compliant magnetic flexible endoscope with diagnostic and therapeutic capability using an actuating permanent magnet in animal studies. They were able to conduct autonomous endoscopic retroflexion with 100% success in 30 attempts without perforation or trauma in pigs. However, diagnostic capability was worse than traditional endoscopy with an average detection miss rate of 21.7% and completion time of 575 s (compared to 5% and 257 s). When looking at lesion targeting alone, the robotic program took on average 251 s to identify lesions compared to only 32 s with traditional endoscopy[65].

In 2020, Formosa *et al*[66] showed the use of a sensor-enabled treaded robotic colonoscope with multiple degrees of freedom which was notable for containing all the functions of a traditional endoscope including direct visualization, channels for insufflation and irrigation as well as a tool port for endoscopy. It also had inertial measurement technology in addition to a magnetometer, motor encoders, and motor current sensors for future autonomous use. *Ex-vivo* porcine results showed locomotion ability up to 40 mm/s[66].

Table 8 Confocal endomicroscopy

Ref.	Study design	Algorithm type	Dataset	Results
André*et al* [58]	Diagnostic model development	CAD using content based image retrieval (CBIR) approach	135 polyps from 71 patients	Accuracy: 89.6% Sensitivity 92.5% Specificity 83.3%
Ştefănescu *et al*[59]	Diagnostic model development	CAD using NAVICAD and a two layer CNN	1035 endomicroscopy images including 725 for training, 155 for validation, and 155 for testing.	Testing decision accuracy error rate of 15.48% (24 out of 155 images)
Taunk *et al* [60]	Feasibility study	CAD using expectation-maximization algorithm	189 endomicroscopy images from 26 patient	Accuracy: 94.2% Sensitivity 94.8% Specificity 93.5%

CAD: Computer-aided diagnosis; CNN: Convoluted neural network.

Table 9 Robotics

Ref.	Study design	Algorithm type	Dataset	Results
Eickhoff *et al*[63]	Prospective, nonrandomized, unblinded feasibility study	CAD using NeoGuide Endoscopy System	10 patients	100% cecal intubation rate. Median time to cecum 20.5 min. 0 complications or adverse effects reported at discharge, 48 h, and 30 d
Pullens *et al*[64]	Randomized control trial with crossover design	CAD using automated lumen centralization	8 expert endoscopists and 10 endoscopy-naïve novices performing endoscopy on a validated colon model with 21 polyps	Novice Accuracy: 88.1% Time to cecum: 8 min 56 s Experts Accuracy: 69% Time to cecum: 13 min 1 s

CAD: Computer-aided diagnosis.

CONCLUSION

Traditional colonoscopy has been shown to reduce colon cancer incidence by more than 80%[67]. The application of artificial intelligence, computer-aided detection, and computer-aided diagnosis to this field provides possibilities for improving an already powerful tool. Namely, the possibility of combining these technologies for real-time endoscopic detection and analysis of lesions overall makes the procedure less operator dependent. With the ability to identify smaller diminutive lesions as non-cancerous, these techniques also offer time and resource savings. Given the widespread use of colonoscopy as a screening test and an aging world population, this potentially translates to billions of dollars in cost reductions and even the possibility of extending screening intervals. Multiple modalities have shown to increase adenoma detection rates and have negative predictive values > 90%, meeting the goals of the ASGE's PIVI initiatives.

However, multiple challenges remain including a lack of large multicenter clinical trials and comparison of computer-aided detection and diagnosis modalities. Additionally, more widespread regulatory approval on government and payer levels is needed. There is still much room for clinical research following software development and more prospective studies evaluating the real-life application of these technologies in the endoscopy suite. Nonetheless, continued development of CADe, CAD and AI in colonoscopy offers patients the possibility of living longer and healthier lives.

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MINIREVIEWS

New hope for hepatitis C virus: Summary of global epidemiologic changes and novel innovations over 20 years

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Abstract

Hepatitis C virus (HCV) is a global health concern associated with significant morbidity and mortality. Before the approval of second-generation direct-acting antiviral agents (DAAs), interferon therapy and liver transplantation constituted the mainstay of treatment. The introduction of well-tolerated oral DAAs in late 2013 has revolutionized HCV management with over 95% cure rates. The predominance of HCV-related liver transplantations has declined following the widespread approval of DAAs. Despite the unparallel efficacy observed among these novel therapies, pharmaceutical costs continue to limit equitable access to healthcare and likely contribute to the differential HCV infection rates observed globally. To reduce the burden of disease worldwide, essential agenda items for all countries must include the prioritization of integrated care models and access to DAAs therapies. Through transparent negotiations with the pharmaceutical industry, the consideration for compassionate release of medications to promote equitable division of care is paramount. Here we provide a literature review of HCV, changes in epidemiologic trends, access issues for current therapies, and global inequities in disease burden.

Key Words: Hepatitis C virus; Direct-acting antivirals; Global health; Liver transplantation; People who inject drugs; Liver cirrhosis

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Core Tip: Hepatitis C virus (HCV) is a global health concern associated with significant morbidity and mortality. The introduction of well-tolerated oral direct-acting antiviral agents in late 2013 has revolutionized HCV management with over 90% cure rates. Equitable access to these agents remains an important issue, given that countries with the greatest burden of disease have the least access to curative therapies. Here we provide a literature review of HCV, changes in epidemiologic trends, current therapies, and highlight inequities in the burden of disease and access to treatment.

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INTRODUCTION

Hepatitis C virus (HCV) is a blood-borne RNA virus first described in the 1980s. During the acute phase of the infection, a large proportion of individuals are asymptomatic, contributing to increased spread and transmission. Thirty percent of individuals clear the infection spontaneously within the acute phase or the first six months[1]. According to 2017 World Health Organization (WHO) estimates, less than 20% of individuals infected with HCV are aware of their diagnosis[1].

HCV infection is a global health concern leading to significant morbidity and mortality worldwide[1]. According to the most recent global statistics, approximately 71 million persons are chronically infected with HCV, comprising 1% of the population[1]. Chronic HCV infection is associated with significant downstream complications, including liver cirrhosis, hepatocellular carcinoma (HCC), and end-stage liver disease[2]. In 2016, the WHO developed a global strategy with the aim of reducing the incidence of HCV by 90% and associated deaths by 65% by 2030[3]. This strategy entails raising awareness, preventing transmission, increasing detection, as well as optimizing access to pharmacotherapies to ultimately ensure equitable division of resources globally[3]. Here we provide a literature review of HCV, changes in epidemiologic trends, access issues for current therapies, and global inequities in disease burden.

GLOBAL EPIDEMIOLOGICAL TRENDS

Globally, the seroprevalence of HCV is highly variable and likely underestimated in low- to middle-income countries due to inadequate infrastructure for population-wide testing. The Global Burden of Diseases, Injuries, and Risk Factors 2010 (GBD 2010) Study estimated > 184 million have been infected with HCV[4]. This study identified Central Asia, East Asia, and North Africa–Middle East as the most endemic regions, with > 3.5% of their populations affected by HCV[4]. Of these regions, Asia carries the highest burden of disease, with more than 100 million HCV-affected individuals across South and East Asia[4]. Evaluation of global epidemiologic trends with a focus on regional prevalence identifies Asian and African countries to have some of the highest reported cases, with Egypt (18%-22%) and Pakistan (4.9%) exhibiting the highest rate of chronic infections. Among high-income countries, Italy (2.5%-10%) remains the most impacted, with as high as 10% of its population reporting chronic HCV infection.

Contaminated needles are considered to be the primary cause of spread in low-to middle-income countries[5]. The population-level spread is seen in countries where infection control practices were historically insufficient. For example, treatment of schistosomiasis, an endemic parasitic infection in Egypt, between the late 1950s to 1980s with intravenous tartar emetic unintentionally contributed to HCV transmission [6,7]. Contaminated needles also account for the majority of new HCV cases in high-income countries. In fact, the highest rates of HCV transmission in the United States are currently seen among people who inject drugs, comprising 23% of new infections (1.75 million new infections in 2015) and 33% of deaths from HCV[8]. Approximately

67% of individuals who endorse a history of intravenous drug use test positive for anti-HCV, indicating current or past infection[8].

In the United States, the estimated prevalence of HCV infection in the non-institutionalized civilian population is 1.0%, corresponding to 2.7 million in 2010[9]. HCV increases the risk of HCC by up to 20-folds, making it the leading cause of HCC worldwide and accounting for approximately 34% of cases in the United States[10]. Globally, HCV-related cirrhosis and HCC together contributed to 399000 deaths in 2016[11]. For decades, HCC had been the fastest-rising cause of cancer-related death in the United States, likely attributed to the HCV epidemic, obesity, and increased prevalence of non-alcoholic steatohepatitis[12]. In 2016 we observed the first decline in the rates of HCC (Relative Rate 0.96; $P = 0.007$), resulting from improved treatments for HCC and increased availability of direct-acting antiviral agents (DAAs)[12]. In terms of HCV-related mortality, since the introduction of DAAs in late 2013, there has been a significant decrease in United States nationwide HCV-related mortality rates compared with the pre-DAAs era. Although cirrhosis and HCC-related mortality continues to increase in recent decades, HCV cirrhosis-related mortality rates markedly declined from 2014 to 2016[13].

In the advent of DAAs, global declines in HCV viremia are anticipated, and over the past decade epidemiologic surveys have demonstrated such a trend[6]. Unfortunately, few direct data exist to establish global trends of HCV burden in the post DAA era [14]. Employing previously validated simulation models, HCV is projected to become a rare disease by 2036 in the United States[15]. Population-based modeling studies employing European data predict that the number of cured HCV patients will exceed the number of viraemic patients[16]. Irrespective of cure, the same researchers foresee a large number of patients remaining untreated, undiagnosed, and developing advanced sequelae of liver disease[16]. Ultimately, it is expected the burden of the disease will shift from patients with viremia to those with virologically cured HCV and expected to reach older age[17].

HCV VIROLOGY AND GENOMIC SEQUENCING

HCV is a single-stranded RNA virus which is easily transmissible *via* microscopic amounts of as little as 0.6 microlitres of blood[18]. It is primarily transmitted through contaminated needles, inadequate sterilization of medical equipment, transfusion of unscreened blood, as well as high-risk sexual practices. We see higher rates of infection among people from low and middle-income countries, in populations who inject drugs, and in men who have sex with men[5,19].

Following exposure, the virus incubates for 2 wk to 24 wk, though only 20% of patients actually develop symptoms[11]. While a minority of patients spontaneously clear the infection within six months, 70% develop chronic HCV. These individuals are at an elevated risk for significant morbidity and mortality, whereby approximately 20-30% will subsequently develop cirrhosis at 20-30 years[5].

This virus was first described in 1989 as a non-A non-B hepatitis. This was discovered after a significant number of post-transfusion hepatitis cases were identified despite what was felt to be adequate testing for the previously identified hepatitis A and B viruses[20]. It was not until Dr. Michael Houghton's team isolated complementary DNA from a "non-A non-B" infected patient, cultivating the isolation of viral RNA, which was ultimately termed 'HCV'[21]. Following the isolation of this strain, Houghton's team were able to develop accurate serological diagnostic tests, including Elisa immunoassays[21]. Unfortunately, the establishment of HCV through antibody testing alone would under detect a quarter of chronically infected patients. These misclassifications resulted from the seroconversion processes, which occur 4-10 wk after transmission, can be circumvented by HCV RNA reverse transcription-polymerase chain reaction testing[22]. Identification of active infection is the primary utility of the HCV RNA testing modality, which is now universally employed. Genomic sequencing of the HCV was completed by Choo *et al*[23] in 1991. This remarkable achievement led to the distinct categorization of HCV by varying genotypes, now numbered from 1-7, with further subcategorizations of related HCV genotypic strains known as subtypes (labelled alphabetically)[22,24]. Despite the description of seven known genotypes, the first six remain the most commonly studied and reported[25]. Genomic isolates of HCV have been well characterized by their geographic variation, which is partly explained by human migration, environmental epidemiologic changes (attempts at eradication of schistosomiasis), and viral evolutions observed across continents[25]. A summary of the HCV genotypes, subtypes, and

geographic variation can be found in Figure 1. Understanding the genotypes, in addition to their geographic variation, is crucial, as interventions are organized for distinct genotype targets. This significant discovery and contribution to science ultimately garnered international recognition, winning Dr. Houghton's team the 2020 Nobel Prize in Physiology or Medicine[26].

OUT WITH THE OLD, IN WITH NEW–NOVEL THERAPEUTICS IN THE TREATMENT OF HCV

The goal of HCV treatment is to cure patients through the eradication of HCV RNA as measured by the sustained virologic response (SVR) at 12 wk after treatment completion[5]. Evaluation of SVR at 12 wk is still considered controversial, where historically, assessment of SVR at 24 wk following treatment completion was considered the gold standard. While high concordance between SVR measured at 12 and 24 wk has been previously demonstrated in trials of pegylated interferon and ribavirin[27], there is less evidence demonstrating the appropriateness of a 12-wk SVR target in the literature for DAAs. A recent systematic review from 2016 did evaluate the concordance of SVR at 12 and 24 wk across two studies of DAAs, demonstrating a reliable correlation of SVR at 12 and 24 wk among multiple populations infected with HCV, including treatment naïve, varying viral genotype, and prior null responders [27]. This evidence was ultimately used to inform future clinical trial design and current recommendations.

Approximately 99% of patients who attain SVR will remain infection-free 5 years later, in the absence of re-infection[28]. Treatment is associated with reduced rates of HCC, liver-related morbidity, as well as liver- and all-cause mortality[5,29,30]. Unless contraindicated, all patients with chronic HCV should be offered treatment. Between 1991 and 2011, combination treatment with pegylated interferon and ribavirin was the mainstay of therapy, achieving SVR rates of up to 40%-50%[5].

DAAs function by targeting proteins involved in HCV replication[29]. The first-generation DAAs for HCV treatments (boceprevir and telaprevir) were approved for use in combination with pegylated interferon and ribavirin in 2011, but have been largely replaced by second-generation DAAs since[29]. The introduction of second-generation DAAs from late 2013 has revolutionized HCV care, achieving cure rates of over 95% among treatment-naïve patients. These interferon-free oral regimens have allowed for major advancements in HCV management, given their ease of administration, much more tolerable side effects profile, and superior efficacy[5,29]. The choice of regimen is guided by HCV genotype, as well as other factors, including previous treatments received and liver cirrhosis[5,29]. Genotypic viral variations add to the complexity of DAA selection. The most desirable therapy would be a pangenotypic option that can be utilized across viral genotypes and subtypes[31]. This was the motive behind the novel NS5A inhibitor, velpatasvir, demonstrated to have 90% SVR at 12 wk across patients with genotypes 1 through 6[32]. Other combination therapies, including glecaprevir/pibrentasvir have also been demonstrated to show pangenoytpic effectiveness[33-35].

Drug-drug interactions (DDIs) are concerning for patients with HCV, especially in the era of DAAs where complex treatment regimens require multiple medications, in conjunction with the other therapies utilized for optimal control of comorbid disease and the side effects of hepatitis treatment itself[31]. The majority of DDIs involve interaction between drugs using the cytochrome P450 enzymes[36]. Careful observation must be given prior to any drug treatment initiation within this population. Often managing teams will include specialist pharmacists responsible for determining the risk for DDIs, approving patients already existing medications, or adding new ones.

Among pregnant women, HCV prevalence estimates range between 0.1% to 8%, depending on the setting and country[37]. However, despite the prevalence of HCV in this population, until recently, the mainstay treatment of HCV, including ribavirin, was considered contraindicated due to the teratogenic and embryotoxic properties of this therapy[37-39]. The introduction of DAAs has opened up questions around the role of DAAs in pregnancy. While there are investigations underway, overall safety data in this population is lacking, and, therefore, treatment with DAAS in pregnancy is currently not recommended in most guidelines[37-39]. For similar reasons, treatment of HCV is also not recommended in breastfeeding women or during lactation[37-39]. Where possible, women of reproductive age should be screened for HCV infection, and if found to have HCV infection, recommendations suggest offering treatment

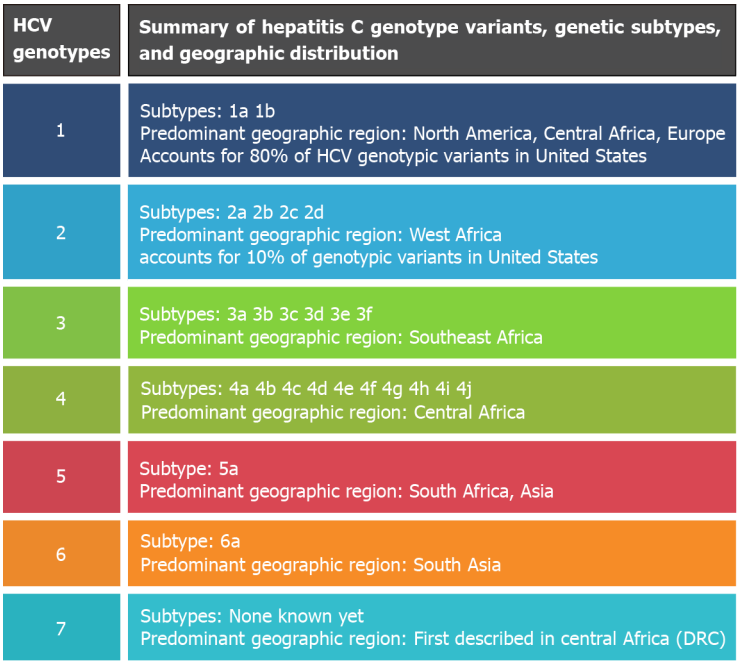


Figure 1 Summary of hepatitis C virus genotypes. HCV: Hepatitis C virus.

prior to conception and pregnancy, or following the completion of pregnancy and lactation in the postpartum period, and screening[38,39].

Compared to existing guidelines for the treatment of HCV or HBV mono-infection are well delineated in both national and international settings, treatment guidelines for HCV/HBV co-infection are less established and remain an area requiring further data [40]. Early reports on the use of DAAs and interferon-based intervention have suggested an increased risk for HBV reactivation with treatment of HCV without concomitant suppression of HBV[41]. However, the clinical significance of this is inconclusive.

While there has been evidence for lower response rates to DAA treatment in HCV populations with parallel injectable recreational drug use, a 2020 study led by Koustenis *et al*[42] offers real-world data that sustained virological response to DAAs is possible in high-risk patients who inject drugs[42]. This is in keeping with established trends revealing similar treatment responses in these higher-risk populations to that of the general population[42]. However, adherence and loss to follow-up remain ongoing concerns among high-risk populations, and during the study, over 10% of patients who inject drugs were lost to follow-up during or after treatment[42]. The risk of being lost to follow-up was correlated with several factors, including younger age and parallel recreational drug use[42].

There have been some controversies as to the safety and efficacy of DAAs for reducing HCC[43,44]. Recent studies have reported that DAAs-based SVR resulted in approximately 50%-80% risk reduction of HCC in patients with cirrhosis[45,46]. Based on the recent expert review, successfully eradicating HCV infection by DAAs confers a protective benefit with reduced incidence of HCC[47]. In terms of HCC recurrence, DAAs therapy was not associated with an overall or early increase in HCC recurrence after complete response to HCC treatment[48].

The availability and access to DAAs worldwide differ by national income and setting[49]. For example, Gilead Sciences has issued licenses that would enable over 100 countries to access generic DAA regimens, and some countries, such as Pakistan and Egypt, have enabled other companies to manufacture unlicensed DAAs in generic format. However, there are disparities among low-income countries, and several countries have not been registered for access to DAAs[49]. Lack of global funding and license provision may present potential barriers[49]. Among higher-income countries, such as countries in Europe, Japan, and the United States, overall generic drug competition among manufacturers has significantly lowered drugs prices, which has improved accessibility[49].

In the table below, we summarize drugs within each category based on their target site of action, and the HCV genotype for which they are employed[29] (Table 1).

Table 1 Classification of direct-acting antiviral agents[29]

Class	Drug name	HCV genotype
NS3/4A inhibitor	Boceprevir[1]	Genotypes 1a and 1b
	Telaprevir[1]	
	Simeprevir	
	Asunaprevir	
	Paritaprevir	
	Grazoprevir	
	Glecaprevir	
	Voxilaprevir	
NS5A inhibitor	Daclatasvir	All HCV genotypes
	Ledipasvir	
	Ombitasvir	
	Elbasvir	
	Velpatasvir	
	Pibrentasvir	
NS5B polymerase inhibitor: Nucleoside analogs	Sofosbuvir	All HCV genotypes
NS5B polymerase inhibitor: Non-nucleoside analogs	Dasabuvir	Genotypes 1a and 1b

[1]First generation direct-acting antiviral agents.
HCV: Hepatitis C virus.

Considerable efforts have focused on the design and development of a HCV vaccine. Challenges resulting from differential rates of transmission among distinct subgroups, in conjunction with the notable variability in treatment access, acceptance, and compliance, could be prevented the development of an effective vaccine. Unfortunately, many barriers have prevented its development. Challenges to the generation of live-attenuated inactivated whole virus for HCV stems largely from our inability to culture the virus effectively[50]. In the rare instances this has been accomplished, the cultured strains exhibited adaptive mutations, which increased replication in vitro [50]. These processes become further complicated by the enormous genetic diversity observed with HCV, with 7 known genotypes and more than 80 subtypes. Ultimately, major barriers to vaccine development include the significant genetic variability between HCV strains, challenges in vaccine production and testing models, as well as our incomplete understanding of the innate protective immune responses to HCV[50].

LIVER TRANSPLANTATION IN THE CONTEXT OF HCV

Prior to the approval of second-generation DAAs, interferon treatment and liver transplantation constituted the mainstay treatment options for liver disease attributed to HCV infection. HCV was previously identified as the leading cause of liver transplantation waitlist additions and surgeries over the last two decades in the United States[51-53]. However, newer data suggest that the predominance of HCV-related liver disease-related transplantations has declined following the widespread use and approval of second-generation DAAs[54]. More recently, HCV-related liver disease has been replaced by alcoholic liver disease as the leading cause of liver transplantation in the United States[51]. Between 2012 and 2015, HCV accounted for 28% of liver transplantations on average[51], by 2016, following the use of DAAs, 18% of liver transplantations were attributed to HCV-related liver disease[51].

Without curative therapies including liver transplantation, re-infection with HCV occurs in high frequency and commonly results in significant liver graft destruction, resulting in poor patient survival[55]. Approximately 80% of patients will develop allograft hepatitis within two years of liver transplantation without eradication, and 20% of those patients progress to advanced fibrosis or cirrhosis within five years of

transplantation[56]. Of note, cholestatic hepatitis C, which can occur in up to 5% to 10% of patients within one year of liver transplantation, can be rapidly progressive and life-threatening[55]. For this reason, many eligible patients will undergo treatment with DAAs prior to or following liver transplantation, ideally before recurrence[55].

Although the optimal timing of DAAs in the context of liver transplantation is still to be clarified, many eligible patients receive treatment prior to transplantation. The overall low burden of DDIs with widely used immunosuppressants poses no contraindication for use in the post-transplantation period[55]. The advantages of early or pre-transplantation viral eradication can include improved overall liver function, quality of life and liver-related complications, potential avoidance of the need for liver transplantation, and lower rate of post-transplantation infection recurrence[56]. However, pre-transplantation eradication can increase wait times for transplantation by limiting access to HCV-positive donor livers and through the improvement of liver function to the degree that de-prioritizes patients on organ transplantation wait lists [56]. This concept has been referred to as 'Model for End-stage Liver Disease (MELD) purgatory' or 'MELD limbo'[55].

The financial burden of liver transplantation remains considerable and is almost exclusive to high-income countries. In Canada, the average cost of liver transplantation in Ontario is $121732 CAN, and in the United States, the average cost is estimated to be around $163438 USD, including follow-up care and medication-related costs. This is compared to other Organization for Economic Cooperation and Development countries, where costs have been estimated at a mean of $103548 USD[57,58]. On average, the out-of-pocket expense for liver transplantation in the United States was noted to be $476.60 USD[59]. Approximately half (53.9%) patients required personal savings and credit (25%) to offset the costs. When compared to patients undergoing kidney transplantation, liver recipients were more likely to rely on alternative sources of funds, including personal loans from friends or family members, retirement funds, and community-based fundraising campaigns[59]. It is anticipated that the burden of financial costs will proportionally reduce with the lower rates of required transplantation with novel therapeutics such as DAAs.

GLOBAL INEQUITIES IN ACCESS TO CARE: THE PHARMACEUTICAL DILEMMA

The global burden of HCV is disproportionately higher among low and middle-income countries[60]. Previous epidemiologic studies from 2013 estimated 12% of globally prevalent cases lived in countries with reliable access to HCV-targeted therapies[4]. Estimates of global disease burden are directly impacted by disease screening practices. Limitations in public health funding among low- and middle-income countries is a barrier to screening and ultimately prevent widescale testing. This leads to the underestimation of HCV prevalence. It is currently estimated that 41% of the world's population lives in countries with no public health funding for the screening and management for viral hepatitis B and C[60]. Overall, limitations in public health funding and infrastructure, organization and costs of screening, expensive of therapeutics, shortages of highly specialized clinicians, and political prioritization of other infectious diseases such as HIV and tuberculosis, all contribute to the barriers inequitable access to care[61].

When distinguishing the Millennium Development Goals, prioritization of infectious diseases excluded viral hepatitis as a 'top 10' contributor to global morbidity and mortality[60,62]. The prioritization of other infectious diseases above HCV leads to considerable diversion of resources away from targeting the important health outcomes resulting from hepatitis infection, including cirrhosis and HCC[60,62]. Provided the global estimates of morbidity and mortality of both cirrhosis and HCC resulting from viral hepatitis were combined, viral hepatitis would securely rank among the top 10 infectious diseases, redefining its importance on the global arena and focus of resources[63].

The prohibitive costs of treatment for HCV infection have been well reported. Many countries will prioritize curative treatment for HCV with DAAs to mitigate downstream costs associated with end-stage liver disease[64,65]. Irrespective of savings, the total cost of DAAs are substantial. Current estimates place DAAs in the highest cost category, with wholesale acquisition prices listed between $417 USD (glecaprevir-pibrentasvir) to as high as $1125 USD (ledpasvir-sofosbuvir)[66]. Discrepancies between production and wholesale acquisition exist and contribute to substantial grievances with the pharmaceutical industry. For instance, Daclatasvir production cost

for a 12-wk course is estimated to be between $10-30 USD, yet wholesale acquisition cost is marked up to $ 63000 USD for one 12-wk treatment course[66].

DAA drug costs continue to fluctuate as a result of generic drug competition, local manufacturing, and geography[49]. In Iran, reducing pricing on generic DAAs, such as sofosbuvir/daclatasvir, has been made possible by local production and manufacturing of generic formulations, together with resultant market competition[67]. This described process has been documented in other countries, such as India, Pakistan, Bangladesh, China, Brazil, and Morocco[67]. These market factors have allowed for generic DAAs to be priced at approximately $60 USD, compared to a previously quoted price of approximately $10000 USD a decade prior[67]. Concerns, however, have been highlighted regarding the degree of oversight over generic manufacturing in these settings, which may be balanced against quality assurances based mainly on clinical observations of the safety and effectiveness of these drugs in the community [67].

Globally, the United States pays disproportionately higher costs for DAAs, even when compared to other high-income countries. For instance, the cost of sofosbuvir for 12-wk of treatment is $84000 USD, which is disproportionately higher than European countries such as Spain where the cost is estimated to be $25000 USD for the same treatment course[68]. Such discrepancies reflect the purchasing power single-payer healthcare systems hold when negotiating with the pharmaceutical industry[68].

Despite the recent United States Food and Drug Administration approval of the more affordable pan-genotypic glecaprevir-pibrentasvir through federally funded programs[66], there remains considerable inequity in access to DAAs and treatment with curative intent. This is partially driven by the lack of standardized policies within federally funded insurance such as Medicare or Medicaid to define eligibility for treatment[66]. Some insurers have gone so far as prioritizing patients with advanced liver fibrosis or those with clinical features of severe disease[66]. Variability in access to treatment is a serious problem within the United States, driven largely by limitations within the health infrastructure. Disproportionate allocation of resources has led insurers to enact restrictions on the delivery of HCV medications[68]. Regardless of the power publicly funded healthcare systems may hold, such costs are prohibitive to equitable treatment distribution worldwide.

INNOVATIVE EXAMPLES OF HCV ELIMINATION EFFORTS IN RESOURCE-LIMITED SETTINGS

Despite the advent of safely targeted pharmacotherapies, treatment access, as well as the rates of infection and re-infection, continue to soar in specific subgroups, particularly those with substance use disorders[69]. Novel approaches to reducing HCV transmission and improving access to therapeutics include integrative HCV care models, whereby delivery of care through addiction services can serve as an effective approach to HCV prevention. In fact, the scale-up of both opioid substitution therapy as well as needle exchange programs can contribute to substantial reductions in HCV transmission and prevalence[70].

Within addiction services, a variety of integrated models for improving HCV treatment have been proposed and evaluated. These models include referrals to primary care or harm reduction settings, developing specialty clinics with inter-disciplinary teams, organization of hepatology and addiction providers within primary care settings, utilizing technology to improve access with telemedicine, as well as onsite delivery of DAAs within opioid substitution clinics[71]. Prison-based HCV programs have also been proposed as a method to improve the delivery of HCV treatment among specific vulnerable subgroups. Targeting penitentiary populations provides an opportunity for micro-elimination, given the controlled setting and ease at which delivery of care can be executed, as well as organized upon transfer or release[72]. In the largest study of European inmates with hepatitis, HCV care was arranged for more than 80% of inmates exhibiting antibody positivity, with similar rates of treatment acceptance and compliance regardless of high-risk practices (*e.g.*, injecting drug)[73]. Ultimately this model of care proved effective for treatment delivery and completion [73].

Comprehensive community-based HCV care models ("educate, test, and treat" schemas) have been trialed with success in Egypt, a country that experiences one of the highest global burdens of HCV infection[74]. A study by Shiha *et al*[74] tested the real-world applicability of such models[74]. First, eligible villagers who met inclusion criteria for age were identified and screened for HCV infection using rapid diagnostic

tests[74]. Patients who screened possessive would provide an immediate sample to be tested for HCV RNA, and those who had a positive result would be scheduled for a clinic visit for education and initiation of baseline testing and, later, treatment using DAAs[74]. Patients with cirrhosis were followed up clinically at ELRIAH or satellite facilities during treatment, and among these patients, 5.2% (166 patients) were diagnosed with HCC[9]. As a result, treatment cure was established for 84.6% of the 17137 persons identified across 70 villages, and established this community-based care model as feasible and effective in this setting[74]. The data may suggest that community-based HCV care models can be a feasible model to consider in treating HCV patients with similar characteristics as those in the study. Despite their novelty and demonstrated impact within specific settings, few of these models have been tested in a clinical trial, and none have been directly compared.

FUTURE DIRECTIONS

Despite the unparallel efficacy observed among second-generation DAAs, with SVR of over 95%[75-78], the cost of such treatments continues to limit equitable access to care and likely contributes to differential rates of HCV infection globally. To reduce the burden of disease worldwide, essential agenda items for low-, middle-, and high-income countries must prioritize access to DAAs therapies. We must acknowledge the complexity of the proposed task beyond negotiations for lower prices from the pharmaceutical industry.

Ancillary costs contributing to the safe and effective delivery of HCV care are undoubtedly expensive. These expenses are largely driven by the costs associated with healthcare personnel, as well as diagnostic and laboratory testing. Provider experience (specialized training in hepatology, infectious disease, general internal medicine), technology for fibrotic staging, as well as baseline laboratory measures including transaminase levels, HCV RNA level, HCV genotype, measures of synthetic liver function (glucose, platelets, albumin), and current renal function account for the bulk of health delivery expenses. Within the United States alone, accomplishing this task may require federal level funding to negotiate in a single-payer fashion for the United States population in one pool. Given the United States is a multi-payer system, there no incentive for pharmaceutical companies to provide the bulk prices single-payer systems negotiate such as the United Kingdom.

CONCLUSION

On a global scale, continued commitment to the prioritization of HCV is required to increase its importance on the international agenda, specifically the WHO. Additionally, pharmaceutical companies need to consider providing compassionate release of medications to promote equitable healthcare worldwide, especially given that the highest burden of disease rests within low- and middle-income countries.

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MINIREVIEWS

Impact of modern antiviral therapy of chronic hepatitis B and C on clinical outcomes of liver disease

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Abstract

Chronic infections with the hepatitis B and C viruses have significant worldwide health and economic impacts. Previous treatments for hepatitis C such as interferon and ribavirin therapy were ineffective and poorly tolerated by patients. The introduction of directly acting curative antiviral therapy for hepatitis C and the wider use of nucleos(t)ide analogues for suppression of chronic Hepatitis B infection have resulted in many positive developments. Decreasing the prevalence of hepatitis B and C have concurrently reduced transmission rates and hence, the number of new infections. Antiviral treatments have decreased the rates of liver decompensation and as a result, lowered hospitalisation and mortality rates for both chronic hepatitis B and C infection. The quality of life of chronically infected patients has also been improved significantly by modern treatment. Antiviral therapy has stopped the progression of liver disease to cirrhosis in certain patient cohorts and prevented ongoing hepatocellular damage in patients with existing cirrhosis. Longer term benefits of antiviral therapy include a reduced risk of developing hepatocellular carcinoma and decreased number of patients requiring liver transplantation. This review article assesses the literature and summarises the impact of modern antiviral therapy of chronic hepatitis B and C on clinical outcomes from liver disease.

Key Words: Hepatitis B; Hepatitis C; Nucleotide analogues; Directly acting antiviral therapy; Clinical outcomes; Liver disease

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Core Tip: Hepatitis B and C infection contribute significantly to the global burden of liver disease. With the introduction of modern antiviral therapy, there is now an effective curative treatment for hepatitis C and potent suppressive antiviral therapy for hepatitis B. Antiviral therapy has had a positive impact on liver disease by reducing hospitalisation rates and hence, cost to global health systems. Rates of decompensation, hepatocellular carcinoma and need for transplantation have all been lowered. This review article assesses the literature and summarises the impact of modern antiviral therapy of chronic hepatitis B and C on clinical outcomes from liver disease.

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INTRODUCTION

Chronic viral hepatitis B and C infections remain a significant burden on global health. Modern directly acting antivirals (DAAs) have changed the landscape of hepatitis C virus (HCV) treatment with their high rates of sustained virological response and superior tolerability. HCV has a significant global impact with the World Health Organisation estimating that 71 million people worldwide are chronically infected[1]. Between 15%-30% of patients with chronic HCV infection will develop cirrhosis and its related complications within 20 years. On the other hand, despite the development and wide availability of the hepatitis B vaccine, WHO estimated that the global incidence of chronic hepatitis B virus (HBV) infection was 257 million people in 2015 [2].

Both of these chronic diseases result in substantial economic burden. The mean annual all-cause medical cost for healthcare in an American cohort with HCV was $43891 as compared to $17989 in patients without HCV[3]. A retrospective study which conducted an analysis on the United States Veteran population with chronic HBV, calculated the total cost per patient per year to be $39240 for those treated with nucleoside analogues[4]. Treatment of HCV, particularly in the early stages of fibrosis, significantly mitigates the economic burden from HCV and its resultant hepatic complications[3]. The cost of treatment of HBV has decreased significantly over the decades as Entecavir and Tenofovir disproxil fumurate are now both off-patent. On the international market, the median price of WHO-prequalified generic Tenofovir has been reduced from US$208 per year to US$32 per year in 2016[2]. Cost of HCV treatment with DAAs is significant, ranging from $40000 to $90000 depending on the drug and treatment duration. Thankfully, many countries such as Australia and Canada have heavily subsidised treatment, allowing the widespread use of DAAs.

The advent of modern DAA therapies has revolutionised the treatment of HCV. More patients are willing to start treatment with the promise of high cure rates, shorter durations of treatment and limited adverse effects. Early access to effective curative treatment will have significant long-term impact on the outcomes of HCV related liver disease. Advances in HCV therapy have led to a commitment by the member states of WHO to try to eliminate viral hepatitis by 2030. For HBV therapy, nucleos(t)ide analogues (NA) are potent antiviral agents which are becoming more widely available in low to middle economic countries. NAs suppress viral replication and therefore result in improved long-term clinical outcomes in chronic liver disease by reducing the progression of liver injury to advanced fibrosis, cirrhosis, liver failure and hepatocellular carcinoma (HCC). As a direct consequence, the requirements for liver transplantation are also reduced[5,6].

The aim of this review article is to summarise the current literature regarding modern antiviral treatment of chronic HBV and HCV infection and the effects of therapy on clinical outcomes of chronic liver disease.

EFFECTS OF CHRONIC HEPATITIS B TREATMENT ON CLINICAL OUTCOMES

Prevalence

Globally, chronic HBV is one of the most common infectious diseases[7]. In 2015, the World Health Organisation (WHO) estimated that 257 million people, equivalent to approximately 3.5% of the world's population, were living with chronic HBV[7]. In addition, 2.7 million people were coinfected with HBV and HIV[7]. Chronic HBV is associated with development of complications including cirrhosis, liver failure and hepatocellular carcinoma. HBV is a leading cause of liver cancer worldwide[8] and up to one in four people with chronic HBV will die from liver cancer or liver failure if their HBV is not monitored or treated appropriately[7]. The prevalence of HBV varies widely depending on country and is predominantly determined by age of exposure; approximately 90% of infected infants progress to develop chronic HBV, as opposed to a rate of 5% following exposure of immunocompetent adults to HBV[9]. In 2015, 65% of all infected persons with HBV were located in African and Western Pacific regions [7].

Chronic HBV infection is preventable using a readily accessible vaccine. In 2017 there were 4762 confirmed HBV cases reported in the United Kingdom and of these, 324 were acute cases of HBV[10]. The prevalence of HBV has been reduced through the incorporation of routine vaccination in the immunisation schedule in Australia and United Kingdom. In 2017, there was a 73% self-reported uptake of at least one dose of the HBV vaccine in England[11]. In Australia, there were an estimated 226566 people (0.9% of the population) living with chronic HBV[12]. Additionally, it is estimated that of those living with chronic HBV, only 68% have been diagnosed[12]. In 2020, only 22% people with chronic HBV were engaged with regular care and receiving regular monitoring or antiviral therapy[12]. Furthermore, only 9.3% were receiving antiviral therapy[13]. The national target in Australia is to have 50% of chronic HBV patients engaged with care and 20% receiving antiviral therapy by 2022[12].

Decompensation and mortality

Currently, chronic infection with HBV remains incurable. The goals of modern antiviral therapy for chronic HBV are to (1) improve survival and quality of life by preventing disease progression to cirrhosis, decompensation and HCC; (2) induce virological seroconversion of e-antigen and surface antigen; (3) induce long-term suppression of HBV DNA levels; and (4) induce normalisation of serum alanine aminotransferase (ALT) levels which reflect a biochemical response to therapy[14].

Chronic HBV infection can progress to advanced hepatic fibrosis and eventually, cirrhosis. Hepatic decompensation can occur in the context of cirrhosis or acute flare as a result of HBV reactivation. Decompensation presents clinically with the development of ascites, jaundice, encephalopathy or variceal bleeding. In HBV infection, hepatic decompensation occurs with a high HBV-DNA viral load and is more common in patients who are e-Ag positive[15].

Hui *et al*[16] conducted a study of 96 chronic HBV patients over three years and reported 29% of patients developed decompensation. The most common clinical manifestation of decompensation was ascites (70%) followed by variceal bleeding (34%), jaundice (26%) and hepatic encephalopathy (5%). Almost a third of subjects had more than one feature of decompensation. Das *et al*[17] performed a retrospective cohort study involving 253 HBV-related decompensated cirrhotic patients. They reported a mean 5-year survival rate of 19%; the commonest causes of death included hepatorenal syndrome (32%), HCC (28%), variceal bleeding (23%), liver failure (9%) and hepatic encephalopathy (9%).

Acute-on-chronic liver failure is associated with a high mortality rate and occurs in patients with chronic HBV who have experienced an acute liver decompensation event which results in jaundice, ascites, coagulopathy and/or encephalopathy[15]. Acute liver insults may result from recrudescence of HBV replication, viral superinfection (with hepatitis D, HCV, or hepatitis E virus), acute variceal bleeding, infection and/or alcoholic hepatitis[15]. Studies assessing acute-on-chronic liver failure in patients with chronic HBV infection report that antiviral therapy (including Entecavir and Tenofovir) reduces all-cause mortality[15,18,19].

Antiviral therapy suppresses viral replication and reduces the likelihood of loss of hepatic function. The literature shows that antiviral therapy reduces the probability of decompensation and progression of liver cirrhosis[15] (Figure 1). The use of antiviral therapy in HBV-related liver decompensation results in improvement of liver function, as illustrated by improved MELD scores and patients being delisted for liver

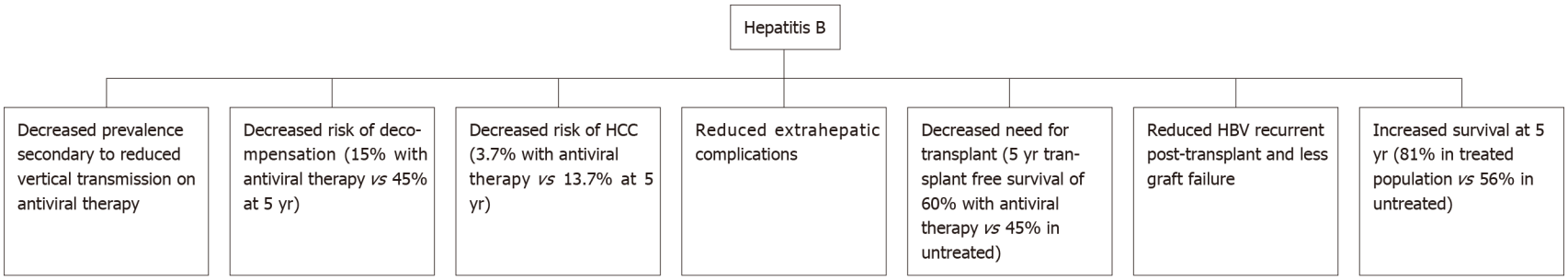


Figure 1 Outcomes of hepatitis B after introduction of antiviral therapy. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus.

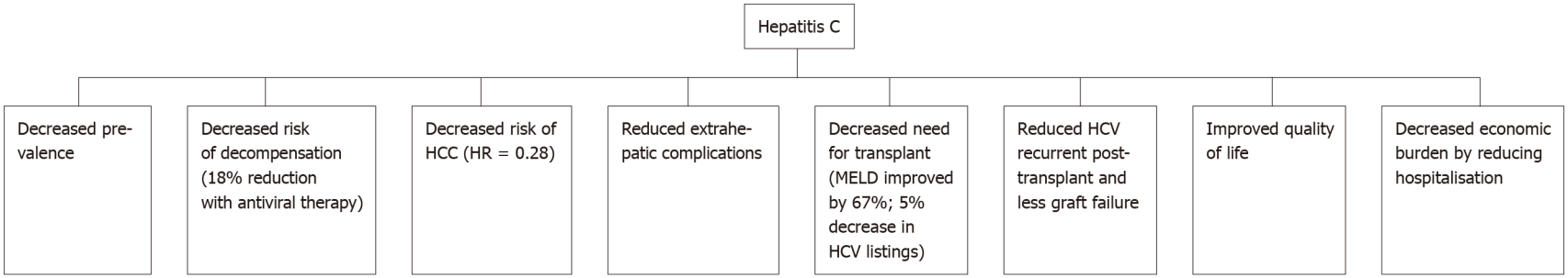


Figure 2 Outcomes of hepatitis C after introduction of antiviral therapy. HCC: Hepatocellular carcinoma; MELD: Model of End stage Liver Disease; HCV: Hepatitis C virus.

transplantation[15]. Therefore, guidelines recommend commencement of antiviral therapy in all cirrhotic patients with HBV, irrespective of viral load level. A Korean study by Kim *et al*[20] demonstrated a 15% probability of developing hepatic decompensation 5-years after commencement of antiviral therapy which was less compared to those who remained untreated and had a 45% probability of decompensation after 5 years. Overall, treatment with antivirals results in significantly improved prognosis in HBV-related cirrhosis as evidenced by 5 year survival rates of 81% in those treated with NAs compared to 56% in the untreated individuals[20].

Nucleos(t)ide analogues such as Entecavir and Tenofovir disoproxil fumurate (TDF) are considered first line agents due to their excellent safety profile, high efficacy and minimal to no risk of resistance developing in treatment-naïve patients[15]. Entecavir induces undetectable viral load in up to 89% of patients with decompensated liver disease after 12 mo[21,22]. Additionally, commencement of antiviral therapy is associated with improvement in prognosis, with a substantial reduction in Model of End stage Liver Disease (MELD) and Child-Pugh (CP) scores after twelve months of

therapy[15]. Several studies have reported a mean improvement of both MELD and CP scores by up to 2 points (compared to baseline) at twelve months in patients who respond to treatment with Entecavir or TDF[21,23-26].

Pegylated-interferon alpha could be considered as an initial treatment option for patients with compensated cirrhosis, with a treatment duration of 48 wk. It is contraindicated in patients with hepatic decompensation. Observational studies which followed up patients for a mean of 84 months and compared treatment with pegylated-interferon alpha *vs* no treatment reported a significant decrease in the risk of HCC, with a cumulative incidence rate of 14% at 5 years in those treated *vs* 23% in untreated patients. There was no difference in all-cause mortality or decompensated liver disease[17,20,27-29].

Several studies have shown that antiviral therapy improves mortality in liver cirrhosis. Suppression of viral replication prevents progression of liver disease and therefore reduces the risk of liver decompensation and HCC, resulting in improved overall survival. The 5-year survival rate in untreated patients with HBV with compensated cirrhosis is 80%-85% *vs* 14%-35% in decompensated cirrhosis[15]. Additionally, a Korean cohort study of 204 patients demonstrated the positive prognostic effect of antiviral therapy in cirrhotic patients with HBV[20]. This study reported the 5-year survival rate in untreated cirrhotic patients was 56%; the survival breakdown based on CP score was 74% for CP-A, 34% for CP-B and 10% for CP-C[20]. For those who were treated with antiviral therapy, the overall 5-year survival rates were 81%; 91% for CP-A, 63% for CP-B and 62% for CP-C[20].

A large study by Jang *et al*[26] which followed 707 patients with HBV-related decompensation for seven years illustrated the long-term benefits of viral suppression in decompensated cirrhotic patients and a trend to benefit in regard to transplant-free survival with antiviral therapy. This study reported a 5-year transplant-free survival of nearly 60% with antiviral therapy compared with 46% in the untreated group. Usually, patients with chronic HBV-related decompensated cirrhosis who are listed for transplantation have a much lower 5-year survival compared with those who undergo transplantation with fulminant HBV infection[26]. In addition, up to 60% of those patients listed for transplantation were able to either delay or negate the need for liver transplantation[26]. However, of the patients with severe hepatic dysfunction in this study, 13% of patients died within first six months of initiating antiviral treatment[26]. Hence, patients with severe decompensation should be considered for liver transplantation because it may take several months of antiviral therapy for liver function to recover.

HCC

Recent studies have reported that the incidence of annual HCC in patients with chronic HBV ranges from 0.9%–1.4% in non-cirrhotic patients and up to 5.4% in those with cirrhosis[30]. The majority of the published literature demonstrates that antiviral therapy with nucleos(t)ide analogues (NAs) reduces the risk of HCC in patients with chronic HBV, after adjusting for known background risk factors including older age, male gender, cirrhosis, family history, HBeAg positive status, elevated HBV DNA and serum ALT levels[13,31,32]. Asian studies that compared data with or without NA treatment reported significant treatment-related reductions in risk of HCC risk by up to 30% in cirrhotic patients and 80% in non-cirrhotic patients[30].

Hosaka *et al*[29] compared 472 Entecavir-treated patients with 1143 untreated patients and found that the incidence rate of HCC at 5 years was 3.7% in the Entecavir group and 13.7% in the control group, after elimination of baseline differences by propensity score matching. Additionally, after adjustment for a number of known HCC risk factors, Entecavir treatment was independently associated with reduced HCC risk[29]. A European multicentre study reported that the HCC risk began to decrease after 5 years of antiviral therapy[33]. The reduction in HCC incidence with treatment was further supported by a Taiwanese nationwide study which assessed propensity score-matched patients on antiviral therapy; those receiving NA therapy had a significantly lower 7 year HCC incidence of 7% compared to 23% in the untreated cohort[13]. The benefit of antiviral treatment was observed in both non-cirrhotic and cirrhotic patients[13].

A large study conducted by Lee *et al*[5] which consisted of a post-hoc analysis of two prospective cohorts from Hong Kong and Korea (n = 818 patients), reported the risk of HCC did not decrease after 5 years of antiviral therapy. This study finding suggests that cirrhotic patients may require longer therapy before a decline in HCC risk eventuates[5]. The higher rates of HCC may also reflect improved mortality rates related to current clinical practice, which translates to more time for HCC to develop [34].

The mechanisms by which antiviral therapy decrease HCC risk may include reductions in the hepatic inflammation and nuclear signaling pathways that lead to neoplastic transformation on a cellular level[35,36]. NAs also reverse fibrosis and the wound-healing response known to be associated with the pathogenesis of HCC[37]. Antiviral therapy may reduce the expression of hepatitis B x-protein to levels that are insufficient for HCC development, or act at a genomic level by preventing integration of HBV DNA into host chromosomes and thus affect its malignant potential[38].

Thus, HCC risk is not completely eliminated with modern antiviral therapy, and there remains a risk of developing HCC even in the context of negative hepatitis B e antigen (HBeAg) and/or negative hepatitis B surface antigen (HBsAg) status[39-42]. Seroconversion of HBsAg is associated with better clinical outcomes and lower rates of HCC incidence[32]. Even so, several studies have illustrated that the risk of HCC remains elevated in treated HBeAg-negative patients for at least 5 years after initiation of NA treatment. Therefore, HCC surveillance should be continued long term, even after seroconversion of HBsAg[31,40,43].

The association between virological suppression and HCC development remains controversial due to conflicting results. In Asian studies, virological suppression is associated with lower rates of HCC – especially in cirrhotics[30,32]. On the other hand, European studies have not consistently reported this association. This difference may be related to variation in patient characteristics such as genotype distribution[6].

A Greek cohort with 321 HBeAg-negative chronic HBV patients treated with Entecavir, reported an HCC incidence of 1.2% at a median follow-up of 1.5 years[40]. The 5-year cumulative HCC incidence rate in Entecavir-treated patients was 9% in cirrhotic patients and less than 1% in non-cirrhotic patients[40]. This result was similar to two other Asian studies[29,44]. Old age was identified to be an independent risk factor associated with increased risk of HCC[40]. A Korean retrospective analysis of 829 patients who had achieved seroconversion of HBsAg and followed up over 3464 patient-years found that the annual rate of HCC was 0.6%; the estimated annual incidence of HCC was 3% in cirrhotic patients and 0.3% in non-cirrhotic patients[39]. Independent risk factors associated with the development of HCC included liver cirrhosis, male gender and age[3] 50 years at time of HBsAg seroconversion[39]. Therefore, HCC surveillance should be recommended to continue in patients who have undergone HBsAg seroconversion if they are cirrhotic or over the age of 50 years, regardless of cirrhotic status[39].

In patients who have undergone resection of HCC, an elevated viral load and absence of antiviral therapy are independent risk factors for recurrence of HCC[45]. Choi *et al*[34] conducted a historical cohort study of 1695 patients treated with Entecavir (n = 813) or TDF (n = 882) after curative intent hepatectomy for early-stage HCC (BCLC Stage 0/A) between 2010 and 2018. During a median follow-up duration of 38 months with continuous antiviral therapy, 33% of patients developed HCC recurrence. A comparison between Entecavir and TDF therapy revealed that TDF was associated with a significantly higher recurrence-free survival, higher overall survival rates and lower rates of HCC recurrence. The 3-year recurrence free survival rates were 73% and 64% in the TDF and Entecavir groups, respectively. TDF was also significantly associated with a lower risk of both early (*i.e.*, within 2-years of resection) (adjusted hazard ratio 0.79, 95%CI, 0.64-0.97, P = 0.03) and late HCC recurrence (adjusted hazard ratio, 0.68; 95%CI, 0.47-0.97; P = 0.03) compared to Entecavir. Whilst the results of this study imply better clinical outcomes associated with TDF in regard to rates of recurrence of HCC, this was an observational study which may be subject to bias and confounding. Additionally, other limitations of the study include missing data, heterogeneity in duration of antiviral therapy treatment (*i.e.*, before or after surgery), and TDF being approved as standard-of-care therapy in Korea in December 2020 which falls within the study period.

Liver transplantation

NAs have been associated with reduced rates of liver transplantation for patients with chronic hepatitis B. A retrospective study included 5374 patients with chronic HBV, 2000 patients were treated with NA (Entecavir) and 3374 patients were treated with Lamivudine in a tertiary centre in South Korea[46]. The study demonstrated that Entecavir was associated with a significantly lower risk of death or transplantation (hazard ratio 0.49, 95%CI 0.38-0.64)[46]. A further analysis of 860 paired cirrhotic patients replicated the finding (hazard ratio 0.42, 95%CI 0.31– 0.57)[46].

Recurrence of HBV post-liver transplantation occurs when circulating HBV virions from extrahepatic reservoirs engraft the new liver. HBV re-infection has been associated with early acute cellular rejection within the first year of liver transplantation[47]. Risk factors associated with HBV recurrence include high level of

viraemia at time of transplantation, HBeAg-positive status, or history of drug resistance to oral HBV antiviral therapy[47,48]. Post-transplant immunosuppression can also induce viral replication and subsequent HBV infection in patients who have received donor liver graft with hepatitis B core antibody positivity[49]. Administration of hepatitis B immunoglobulin (HBIG) to transplant recipients has been associated with lower HBV recurrence and improved survival of 80%, compared to 80% HBV recurrence and 50% survival if no HBIG was given[47,48]. The addition of NA therapy to HBIG in liver transplant recipients has resulted in a further reduction in HBV recurrence and improved prognosis[47]. A study conducted by Cholongitas *et al*[50] which had a median follow up period of 30 mo, reported HBV recurrence was much lower in patients who received HBIG plus NA (Entecavir/Tenofovir) – 1%, compared to those who received HBIG plus Lamivudine – 6% (P = 0.0004).

Extrahepatic manifestations

Extrahepatic manifestations may be observed with acute or chronic HBV infection. It is postulated that circulating immune complexes and viral antigens activate the complement cascade resulting in organ injury, most commonly to the kidneys, vessel wall, skin and joints[51]. A multicentre retrospective cross-sectional study of HBV patients and found that 16% exhibited clinical extrahepatic manifestations - sensory-motor deficiency, sicca syndrome, myalgia, glomerulonephritis and arthralgia arthritis [52]. Other associations reported in the literature include a serum sickness-like syndrome, polyarteritis nodosa, cryoglobulinaemia, lichen planus, Gianotti-Crosti syndrome and Guillain-Barre syndrome. Awareness and recognition of these clinical manifestations are important to facilitate early diagnosis, monitoring and treatment of HBV infection and the associated organ injury.

Infection with HBV is associated with a variety of renal diseases including membranous nephropathy, membranoproliferative glomerulonephritis and rarely IgA nephropathy. A prospective French study by Mallet *et al*[53] monitored the renal function of patients with compensated chronic HBV following treatment with NAs. Treatment with TDF or Entecavir, especially in patients with an initial HBV DNA titre of 100 000IU/mL or greater, resulted in an increased estimated Glomerular filtration rate (eGFR) throughout the follow up period (median of 2.7 years)[53]. Similarly, a recent meta-analysis by Fu *et al*[54] assessed the efficacy and safety of NA monotherapy for HBV-related glomerulonephritis in 7 trials involving 182 participants. They found that antiviral therapy induced remission of proteinuria and increased HBeAg clearance.

Polyarteritis nodosa (PAN) is a multisystem necrotizing vasculitis typically affecting muscular medium-sized and small arteries. Hepatitis B has been observed in 17%-35% of patients diagnosed with PAN, although the frequency is likely decreasing due to HBV vaccination[55]. The American College of Rheumatology (ACR) has established ten criteria for the classification of PAN in patients with a vasculitis and recognises the evidence of HBV infection as one of the diagnostic criteria[56]. Other systemic features of PAN may include cutaneous livedo reticularis, testicular pain, mononeuropathy or polyneuropathy, renal artery aneurysm and unexpected weight loss. In patients in whom PAN is associated with HBV (HBV-PAN), the major focus of therapy is the use of antiviral agents for the treatment of the underlying viral disorder. Immunosuppressive therapy in the form of glucocorticoids may also be used in the short term for control of the systemic inflammation[55]. In several studies, clinical remission of PAN was observed in patients who were treated with antiviral agents and in very few patients who instead received only immunosuppressive treatment[55,57].

Mixed cryoglobulinaemia (types II and III) is caused by immunocomplexes composed of polyclonal IgG in association with either monoclonal or polyclonal IgM. These immunoglobulins precipitate with cold temperature, resulting in purpura, renal disease, arthralgia or arthritis. Cryoglobulinaemic vasculitis can develop in 1-4% of HBV patients. Treatment with NAs has been found to provide significant clinical response, preventing extrahepatic organ complications[58].

EFFECTS OF CHRONIC HEPATITIS C TREATMENT ON CLINICAL OUTCOMES

Prevalence

The WHO estimates that there are up to 71 million people worldwide who are chronically infected with HCV[1]. In Australia, mandatory reporting of HCV diagnosis

has been required since the 1990s. This allows for more accurate assessment of HCV numbers. As of 2017, it was estimated that up to 182,144 people in Australia had HCV. The incidence of new HCV infections has steadily declined since 2000. Government subsidised DAA treatment was made generally available in 2016. At the end of 2018, an estimated 130089 Australians had HCV, a decrease of approximately 50000 patients with the use of antiviral therapy (Figure 2).

In the United Kingdom, the prevalence is estimated to have decreased in recent years by one-third due to DAA treatment. Approximately 118000 people had HCV in 2019 as compared to 174000 in 2015[59]. It is expected that the prevalence will continue to steadily decrease with a corresponding increase in the number of patients who have been successfully treated.

Decompensation and mortality

It is well recognised that chronic HCV places considerable financial burden on the healthcare system, primarily related to presentations with decompensated liver disease. These individuals have prolonged stays in hospital and frequent re-admissions. A United Kingdom study demonstrated that 21.1 d was the mean length of stay in decompensated HCV patients without HCC. This translates to a mean cost of 9120 pounds per year. Within Europe, annual hospitalisation costs are estimated at 8000-20000 euros for decompensated patients in France and up to 28000 euros in the Netherlands. The inpatient hospital burden appears to be greatest within the first year after the first decompensation event[60]. With the aging population, there are increasing numbers of patients presenting to Emergency Departments with HCV-related issues which result in hospitalisation[61]. In the Scottish population, between 1996 and 2013, the total number of HCV-related inpatient admissions with decompensation increased 16-fold[60].

In the United Kingdom-based Extended Access Program cohort, achieving a sustained viral response (SVR) in HCV cirrhotics was associated with a reduction in decompensation rates of 18% within the first 6 mo[62]. Lower decompensation rates result in decreased hospitalisation rates, reduced length of stay and most importantly, mortality reduction[63]. In the Veterans Affairs cohort, achieving SVR in patients with advanced liver disease (pre-treatment FIB-4 scores of > 3.25) resulted in a 74% reduction in mortality risk, after adjusting for baseline variables[64].

A rise in the hepatic venous pressure gradient (HVPG) is the main driver behind cirrhosis-related complications such as variceal bleeding and ascites. HCV eradication is associated with a reduction in HVPG which translates to decreased complications. This was demonstrated in a prospective study by Mandorfer *et al*[65] who measured HVPG pre- and post-treatment of HCV. In patients with subclinical portal hypertension (HVPG 6-9 mmHg), portal hypertension resolved in 63% of patients. In the group of patients with HVPG 10-15 mmHg, portal hypertension resolved in 43% of the cohort. The highest risk group with HVPG > 16 mmHg showed a reduction in HVPG in 35% of patients but portal hypertension did not resolve in any of the patients[65]. This indicates the importance of treating HCV early to achieve SVR and reversibility of portal hypertension prior to development of end-stage irreversible portal hypertension and its related complications.

Quality of life

Chronic HCV infection has clearly defined effects on hepatic fibrosis, cirrhosis and subsequently, increased risk of HCC. There have been many studies looking into the clinical effects of hepatitis C-related liver disease. Patient related outcomes (PROs) or impact on quality of life are equally important measures but harder to quantify. Previous studies have demonstrated that health-related quality of life (HRQOL) is impacted by HCV and HCV causes a more severe reduction in HRQOL than certain chronic diseases such as diabetes[66,67].

Cacoub *et al*[68] investigated the impact of HCV treatment on patient-related outcomes in a large study. Data were derived from 11 multicentre phase 3 clinical trials across a range of countries. These included trials based on interferon therapy, ribavirin-based and DAA therapies. PROs were measured using a combination of four different validated questionnaires to encompass different domains including physical, emotional and social impact as well as work productivity, fatigue and functional well-being. Patients were asked to fill in the questionnaires at baseline, during treatment and post-treatment while being blinded to their HCV RNA levels.

This study demonstrated that modern antiviral therapy was associated with improvement in PROs as early as four weeks after initiation of treatment[68]. By week 4, average PRO scores had improved by 6%, with the greatest improvements in the domain of emotional wellbeing and worry. This pattern continued throughout

treatment course (increased by 8%) and was sustained up to SVR-24 (increased by 10%), becoming more pronounced with time. PROs were significantly better in the non-interferon, non-ribavirin group[68]. This provides additional support for the superiority of modern DAAs over older treatment regimes.

A systematic review by Younossi *et al*[69] also demonstrated that HCV infection negatively impacted patients' HRQOL. In particular, scores were lower in the mental health domains as compared to physical health. The prevalence of depression in HCV patients was estimated to be 24% compared to 17% in the non-HCV control group. Based on this data, the risk of developing depression in patients with HCV is substantially increased (RR = 2.30)[69]. In addition to the physical benefits derived by HCV clearance, the significant mental health improvements are also an important reason to treat HCV patients as early as possible.

HCC

HCC is the second leading cause of cancer-related death worldwide. Early on in the post-DAA treatment era, there was initial concern about an unexpected increase in the incidence of HCC after achieving viral clearance. These results were later disputed by other studies which had longer follow-up periods and larger cohorts with control groups. A cohort study by Kanwal *et al*[70] demonstrated that SVR was associated with a significantly lower risk of HCC as compared to patients who remained infected (0.90 *vs* 3.45 HCC per 100-person years; adjusted HR = 0.28). Two large cohort studies by Ioannou *et al*[71] and Singer *et al*[72] also showed a significantly reduced risk of HCC following SVR with adjusted HR = 0.84 and 0.29, respectively. Cheung *et al*[62] carried out a prospective study involving 406 patients with decompensated cirrhosis and demonstrated that the incidence of HCC was 4% within the first 6 months of DAA treatment. This was equivalent to the matched control group with untreated patients. The evidence now overwhelmingly favours the absence of any increased risk of HCC following SVR[73,74].

Post-treatment studies from the interferon era have shown that regression of fibrosis is a gradual process that can take years. As fibrosis assessment is mostly performed using non-invasive markers now, there is limited data assessing fibrosis regression post SVR with DAAs using the gold standard of liver biopsy. One small study showed that cirrhosis resolved in 7 out of 14 patients following DAA treatment[75]. A large retrospective Brazilian study of 400 patients reported that liver stiffness measurement (measured with Fibroscan) decreased significantly post-SVR (13.6 kPa *vs* 10.2 kPa, $P < 0.001$) with 42% of the treated cohort experiencing a reduction of LSM by at least 30% [76]. Thus, early viral clearance offers advantages of prevention and regression of hepatic fibrosis.

Although treatment with DAAs does not confer additional risk of HCC development, it is still important to note that patients remain at risk of HCC even following SVR. Multivariate analyses from several studies have identified risk factors for HCC occurrence post DAA treatment. These include advanced liver fibrosis (F3 or F4), older age and male sex[77,78]. Therefore, it is imperative that patients with these risk factors should continue to undergo HCC surveillance even after successfully achieving viral clearance.

Liver transplantation

HCV is one of the leading reasons for liver transplantation in the Western world. Twenty-four percent of liver transplants performed in Europe between 1999 and 2009 were HCV-related[79]. The recurrence of HCV post-transplant was also an issue leading to accelerated graft dysfunction and eventual failure. DAA treatment can reduce the need for transplantation by reducing decompensation rates. A pooled analysis of 800 decompensated cirrhotic patients demonstrated that up to 60% of patients had an improvement of their MELD score after treatment[62]. In the United Kingdom, the introduction of treatment with DAAs has resulted in a decreased number of HCV-related cirrhosis patients being listed for transplant. The rate in 2013 of new registrations with HCV-related cirrhosis was 10% and this reduced to 5% in 2016 ($P < 0.001$) In this cohort, the median UKELD for HCV remained stable at 55 in the pre and post-DAA era[62,80,81]. There has been concern that treatment of HCV patients on the transplant waitlist will decrease their MELD score, reducing their chances of receiving a transplant. Despite the reduction in their MELD score, some of these patients still require a transplant as they are experiencing symptoms of decompensation. Ironically, the lower MELD score makes it less likely for them to receive a transplant, thus leaving this group of patients in "purgatory". This data indicates that the threshold which physicians were using for listing patients had not changed over time and that the concept of entering "MELD purgatory" was not

occurring in the United Kingdom[81].

Other centres in Europe have reported an improvement in liver function of HCV patients on the transplant waitlist following DAA treatment to the point where up to 1 in 4 patients can be removed from the waitlist or delisted. This decline in listing numbers is similar to that noted a decade ago after the introduction of antiviral therapy for hepatitis B. In an era where there is a chronic shortage of donor organs and an increasing need, early treatment with DAAs is beneficial in reducing the number of patients needing to progress to transplant[82,83].

HCV recurrence in the graft was a common problem post-transplant in patients with HCV. This contributed to accelerated fibrosis progression due to an immune incompetent state, resulting in graft dysfunction and eventual failure. With the introduction of DAAs, treatment of HCV recurrence in the post-transplant setting now prevents graft loss and the need for re-transplantation in this cohort[82,83].

Extrahepatic manifestations

HCV infection has an impact not only on the liver, but also systemically. A meta-analysis of 22 studies demonstrated that HCV infection increases the risk of cardiovascular mortality, with a two-fold higher risk of subclinical carotid plaques amongst HCV patients. HCV patients also have a higher risk of peripheral artery disease, cardiovascular events and cardiovascular mortality[84]. These changes are theorized to be due to systemic inflammation and release of inflammatory cytokines within the body caused by the virus. Based on this theory, treatment of HCV should result in a reduction in risk of extrahepatic manifestations.

A prospective Italian study by Nevola *et al*[85] evaluated HCV patients before and 24 wk after treatment with DAAs. After achieving SVR, patients demonstrated an improvement in fasting blood glucose levels and reduced insulin resistance, regardless of the fibrosis state. In patients with impaired fasting glucose pre-treatment, 32% had normal blood glucose levels following SVR. Forty-five percent of diabetic patients experienced an improvement in glycaemic control, with some patients managing to reduce or cease their diabetic medications[85]. This indicates that HCV clearance is associated with a significant change in glucose metabolism, independent of baseline BMI. This has also been confirmed by other studies[86-90].

Changes in lipid profiles also occur following SVR, commonly manifest with an increase in LDL levels. The effect on HDL and triglycerides is less consistent across studies[91-95]. Lipid disturbances require closer monitoring in patients who have achieved viral clearance. Although lipid profiles may deteriorate following SVR, renal function improves. Patients in the Nevola cohort experienced an improvement in eGFR by 10% compared to baseline, regardless of the fibrosis status[85].

Depression is co-morbid with HCV infection in up to one-third of cases. Patients are often marginalized and experience psychosocial stigma due to their positive HCV status. From a physiological standpoint, it is thought that the systemic inflammation caused by the virus and the invasion of HCV into the nervous system results in disruptions of neurotransmission pathways. Moez *et al*[96] used the Beck Depression inventory scale to prospectively assess change post HCV treatment in their cohort of Egyptian patients and found a significant decrease in scores 1 month and 3 months post-SVR which correlated with an improvement in mood symptoms.

CONCLUSION

The use of NAs in HBV and DAAs in HCV has resulted in substantial benefits to patients who suffer with these chronic diseases. The therapeutic benefits gained from treatment encompass not only manifest as improvements in physical and mental health but also quality of life and economic health burden. It is hoped that there will be effective curative treatment for HBV in the coming years. Although there has been a decrease in the incidence of chronic HCV with the introduction of DAAs, there is still much work to be done before achieving the WHO's target of HCV eradication by 2030. Reaching this target will require further screening, especially in at-risk groups, and more widespread subsidised use of DAAs worldwide.

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MINIREVIEWS

Neurological and psychiatric effects of hepatitis C virus infection

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Abstract

Hepatitis C virus (HCV) infection is widespread and affects 71 million people worldwide. Although hepatic manifestations are the most frequent, ranging from chronic hepatitis to cirrhosis and hepatocellular carcinoma, it is also associated with several extrahepatic manifestations. Infected patients may present non-specific neurological symptoms, regardless of the presence of liver cirrhosis. Several pathogenetic mechanisms underlying neurological symptoms have been hypothesized: neuroinvasion, immune-mediated damage, neurotransmitter alterations and cryoglobulinemia. Alterations of the central nervous system include cerebral vasculopathy, acute or subacute encephalopathy and inflammatory disorders. HCV infection may be responsible for neuropathies, of which the most frequent form is symmetrical axonal sensory or sensory-motor polyneuropathy which causes loss of leg sensitivity and weakness. Up to 50% of patients with HCV infection may experience cognitive decline and psychological disorders, such as depression and fatigue. HCV associated neurocognitive disorder is independent of the presence of liver cirrhosis and affects different domains than in patients with hepatic encephalopathy. It can be studied using specific tests that mainly explore executive functions, verbal learning and verbal recall. These disorders significantly reduce the quality of life. The new antiviral therapies improve the extrahepatic symptoms of HCV infection and their success depends on the achievement of sustained virological response. However, the effect of therapy may differ depending on the type of organ involved; neurological symptoms can be irreversible if there is organic liver damage. The aim of this review is to provide a critical overview of physiopathological mechanisms, diagnostic and therapeutic strategies of the neurological and psychiatric effects of HCV infection.

Key Words: Hepatitis C virus; Neurological disorders; Psychiatric disorders; Antiviral therapy; Quality of life; Hepatic encephalopathy

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Core Tip: The aim of this review is to provide a critical overview of neurological and psychiatric disorders in patients with hepatitis C virus infection, the underlying pathogenetic mechanisms and the effect of new direct-acting antivirals on extrahepatic symptoms. The main clinical and pathogenetic differences to hepatic encephalopathy and the different diagnostic strategies used in these two conditions are reported.

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INTRODUCTION

Hepatitis C is caused by hepatitis C virus (HCV), an RNA virus belonging to the family Hepadnaviridae. It has been estimated that there are 71 million patients with HCV infection worldwide[1] and it is an important cause of liver-related morbidity and mortality[2].

Despite the prevalent hepatic involvement, HCV infection is now considered a systemic infection due to increasing evidence of the ability of the virus to enter and replicate in major cellular systems[3].

HCV infection has been associated with more than 30 extrahepatic manifestations [4], some of which are a consequence of the proliferation of B lymphocytes with consequent monoclonal and polyclonal production of antibodies showing the activity of the rheumatoid factor or cryoglobulins[5].

Extrahepatic symptoms can prevail over hepatic symptoms or aggravate them[3] and can involve cardiovascular, joint, dermatological, renal and neurological systems.

Hepatic encephalopathy is the most typical manifestation of central nervous system involvement in patients with liver cirrhosis. The hypothesis that chronic HCV infection can itself cause brain dysfunction derives from evidence from patients without cirrhosis or cryoglobulinemia who frequently have a wide range of non-specific neurological symptoms[2].

Up to 50% of non-cirrhotic patients with HCV develop neurological and psychiatric symptoms whose manifestations do not correlate with the severity of liver disease or with viral replication rate[3].

Thus, in the diagnostic work-up of a patient with neurological and psychiatric symptoms, physicians should also consider HCV infection as a possible etiological factor[3].

Finally, it should be considered that the presence of HCV in an extrahepatic and immune-privileged site could be a potential source of late relapse after antiviral therapy[2].

PATHOGENESIS

The ways in which HCV causes the occurrence of neurological and psychiatric symptoms are different and several mechanisms have been hypothesized: Neuroinvasion and direct damage: Several studies have shown that the nervous system is a permissive site for viral replication, as confirmed by the presence of intermediate replicative forms of HCV-RNA and viral proteins at this level[3].

Furthermore, the presence of HCV in the cerebrospinal fluid of patients with cognitive decline has been demonstrated through gene sequencing techniques[2], but HCV-RNA levels are 1000-10000 times lower than those found in the liver[6].

However, the viral sequences of E1 and 5′ untranslated regions found in the central nervous system are different to the hepatic sequences, suggesting an independent life of the virus in the central nervous system[3,6].

In line with this hypothesis, cerebral viral quasispecies share genomic sequences with those found in lymphoid tissues and peripheral blood mononuclear cells, but not with hepatic cells[3].

These data suggest the hypothesis that peripheral blood mononuclear cells can mediate the entry of virus into the central nervous system through a mechanism called "trojan horse"[7].

Therefore, the virus first infects peripheral monocytes and then crosses the blood-brain barrier after infection of brain microvascular endothelial cells[6]. In this way, virus, immune cells, cytokines and chemokines can enter the central nervous system causing immune brain activation[8,9].

It should be noted that microvascular endothelium cells are the only cells in the central nervous system that express HCV receptors; so, they play a critical role in allowing virus entry in this site[10].

CD-81, SR-BI and claudin 1 are expressed on epithelial and endothelial cells of different tissues and it has been shown that antibodies against these antigens inhibit virus entry into cell lines derived from cerebral endothelium *in vitro*. Therefore, they can represent the target for HCV entry in brain endothelial cells[10,11].

Other studies have shown that Apolipoprotein E (ApoE) also plays an important role in this process. In fact, anti-ApoE antibodies can neutralize infection in cell lines derived from cerebral endothelium[6].

Therefore, although normally microglia do not express the molecules necessary for HCV entry, the virus evades this obstacle by directly infecting peripheral precursors and altering the selective filter of the blood-brain barrier. This mechanism is similar to that described for HIV[12,13].

Confirming this hypothesis, immunohistochemical and molecular techniques have shown that astrocytes and microglia are target cells for HCV infection[12,14].

Cells with positivity for HCV NS3, also express CD-68 which is a marker of glial cells. When RNA of these cells is amplified, the result is expression of both microglia markers and pro-inflammatory genes such as IL-12, IL-8 and TNF alpha[14]. Moreover, the activation of infected microglia cells results in an increased production of excitatory amino acids that can induce neuronal death through excitotoxicity[15].

Metabolic and neurotransmission alteration: Magnetic resonance spectroscopy allows measurement of the concentration of certain metabolites in specific brain regions. Choline is a metabolite expressed by glial and proliferating cells and is used as a marker of inflammation and of the synthesis and turnover of cell membranes; N-acetyl aspartate is a neuronal metabolite and a marker of neuronal integrity, so its decrease is indicative of neuronal loss or dysfunction; myoinositol is produced only by glial cells and increases with glial proliferation, called gliosis, or with interruption of myelin[2,16].

Several studies have shown an increased level of choline and myoinositol, both in cerebral white matter and basal ganglia, and a reduction of N-acetyl aspartate level in basal ganglia. These findings are suggestive of glial activation and macrophage infiltration and support the hypothesis of neuroinflammation.

Neurotransmission studies using single photon emission computed tomography (SPECT) demonstrated an alteration of serotonergic and dopaminergic neurotransmitter systems in the midbrain and basal ganglia of up to 60% in HCV patients[17,18]. This can be related to high peripheral cytokine levels, particularly IL-1 and IL-6, which can negatively interfere with neurotransmitter systems and impair memory and executive functions[2,19].

Finally, perfusion studies have shown increased perfusion in the basal ganglia compared to cerebral cortex; this report suggests a vascular response secondary to immune activation due to neuronal invasion, confirming the hypothesis of neuroinflammation[20].

This picture is typical of other inflammatory conditions such as multiple sclerosis [21].

Overall, these studies have shown abnormalities in dopamine and serotonin signaling, neuronal hypometabolism and neuro-inflammation in some patients with chronic HCV infection.

Cryoglobulinemia: cryoglobulins are immunoglobulins able to bind to each other at temperatures below 37°C, causing organ damage through two main mechanisms. The first mechanism, associated with type I cryoglobulinemia, consists of "vascular sludging" which is a hyperviscosity syndrome; the second mechanism, typical of type II and III cryoglobulinemia, is a mechanism of immune-mediated vasculitis[22].

One of the consequences of HCV infection is mixed cryoglobulinemia (type II and III) which is characterized by the precipitation of immune complexes. Type II and III cryoglobulinemia are due to an increase in cryoglobulin production by B lymphocytes clones.

HCV lymph-tropism is the first step in inducing cryoglobulinemia; in fact, the E2 protein of the viral envelope interacts with the surface protein CD-81 of B lympho-

cytes, inducing proliferation and production of IgM monoclonal antibodies[23,24].

The characteristic pathological sign of cryoglobulinemia is leukocytoclastic vasculitis which affects medium and small blood vessels with transmural fibrinoid necrosis, thrombotic lumen occlusion and infiltration of polymorphonuclear cells. The consequence of vasculitis is ischemic tissue damage[4]. This condition is now referred to as HCV-associated cryoglobulinemic vasculitis (HCV-associated CV).

CENTRAL NERVOUS SYSTEM DISORDERS

Cerebrovascular disease

Acute and chronic cerebral vasculopathy is more frequent in patients with HCV than in the general population and HCV-RNA level correlates positively with the risk of cerebrovascular death[25].

Acute events such as stroke, transient ischemic attack, lacunar syndrome and, rarely, bleeding, have been reported in patients with HCV infection, in some cases representing the initial clinical manifestation of infection itself[26].

Several retrospective and prospective studies have shown an association between HCV infection and risk of stroke, which can occur at a lower age and without the classic cardiovascular risk factors[25].

Several responsible mechanisms have been hypothesized.

Chronic systemic inflammatory state can be responsible for arterial remodeling that precedes the development of carotid plaque[27], while the locally active presence of virus can cause plaque instability with erosion, rupture and thromboembolic event [28].

Furthermore, chronic HCV infection predisposes to metabolic imbalances; in particular, the virus can alter glucose homeostasis and lipid metabolism in a pro-atherogenic sense[29]. The association between infection and development of type II diabetes mellitus is well documented in the literature[6].

Mixed cryoglobulinemia in the brain can affect vasa nervorum and cerebral arterioles causing occlusive cerebral vasculopathy[2] and they constitute an independent risk factor for carotid plaque formation[4].

Finally, in some patients the ischemic event may be secondary to ANCA or antiphospholipid antibodies which can cause a non-cryobobulinemic vasculitis[4,30].

Acute or subacute encephalopathy

Chronic HCV infection can be responsible for acute and subacute involvement of white matter when associated with cryoglobulinemia or systemic vasculitis[4].

This encephalopathy is clinically characterized by cognitive decline, confusion, altered state of consciousness, dysarthria, dysphagia and incontinence as a result of widespread involvement of cerebral white matter in patients with cryoglobulins or circulating anticardiolipin antibodies[4].

This condition could be due to ischemic damage and this hypothesis is confirmed by the presence of small subcortical and peri-ventricular white matter lesions found on magnetic resonance imaging[4]. In addition, diffuse and severe changes in supratentorial and infratentorial white matter highly suggestive of cerebral vasculitis, have been observed in patients with systemic vasculitis[31].

Inflammatory disorders

HCV infection has been associated with the onset of central nervous system inflammatory diseases which can occur in different clinical scenarios.

In the literature, cases of rapidly developing leukoencephalitis clinically characterized by cognitive decline, tetraparesis and aphasia, and histologically by perivascular infiltrates of T lymphocytes and microglia nodules have been reported.

Fatally progressive encephalomyelitis presenting with epilepsy, motor, sensory and autonomic deficits and characterized by loss of neurons and perivascular infiltration of lymphocytes in the brainstem and cervical spinal cord may occur[32,33].

The hypothesized pathogenetic mechanism is that of immune-mediated damage rather than direct damage induced by virus[4]. Confirming this hypothesis is the response to steroid therapy in cases of acute disseminated encephalomyelitis, a post-infectious autoimmune disease characterized by immune-mediated damage of cerebral and cerebellar white matter and clinically characterized by alterations of consciousness, psychomotor agitation, focal neurological symptoms and urinary retention[3,34].

Chronic HCV infection can also induce myelitis due to antibody-mediated autoimmune demyelination. Transverse myelitis is a common manifestation of this group of disorders in which the immune-mediated mechanism of damage is confirmed by anti-HCV antibodies but not the presence of viral genome in cerebrospinal fluid. The prevalent symptoms are autonomic, sensory and motor dysfunction, sensory ataxia and spastic paraplegia[4].

PERIPHERAL NERVOUS SYSTEM DISORDERS

During HCV infection, a high prevalence of peripheral neuropathy has been reported, which involves 40% to 75% of patients[6].

This finding is more evident in patients with mixed cryoglobulinemia, in whom fascicular ischemia and axonal degeneration are observed as a result of vasculitis of the vasa nervorum or necrotizing arteritis of medium-sized vessels[35]. In fact, up to 60% of patients with cryoglobulinemia present peripheral neuropathy, while a smaller percentage presents involvement of the central nervous system (6%) due to direct involvement of cerebral arterioles[4].

However, peripheral neuropathy has been demonstrated in subjects who do not have mixed cryoglobulinemia. In these cases, immune complexes or autoimmune mechanisms induced by virus and dependent on T lymphocytes, may play a role in inducing vascular and perivascular inflammation[2,36].

In any case, the pathogenesis is indirect and predominantly inflammatory as the virus does not invade peripheral nerves or muscle tissue[6].

Sensory or motor axon degeneration disrupts communication between the central and peripheral nervous systems. Motor damage can be responsible for weakness, while sensory damage can cause paresthesia, hyperalgesia, allodynia, pain and numbness. Autonomic damage can result in organ damage[6].

The most frequent form of peripheral neuropathy is symmetrical axonal sensory or sensorimotor polyneuropathy which is typically characterized by loss of sensation and weakness in the lower leg region[37].

Asymmetrical forms include small fiber sensory polyneuropathy which is the most common clinical manifestation and is characterized by tingling, burning feet and restless legs syndrome. In more advanced cases, neuropathy can affect larger caliber fibers (sensory polyneuropathy of the large fibers) which presents with loss of sensation, paresthesia, cramps and numbness[38,39].

Other clinical presentations are mononeuropathy and multiple mononeuropathy, which involves non-contiguous nerves. Cranial nerves are usually spared from this phenomenon, although there have been reports of abductor, facial and trigeminal nerve involvement[4].

Rare forms of neuropathy are pure motor polyneuropathy and autonomic neuropathy[40,41].

NEUROPSYCHOLOGICAL DISORDERS

Up to 50% of patients with chronic HCV infection has neuropsychiatric symptoms, "brain fog", fatigue and impaired quality of life, regardless of the severity of liver disease[6,13]. These alterations can interfere with daily activities and with the ability to maintain independence[15].

While it is possible that psychiatric symptoms can contribute to cognitive decline in these patients, it appears unlikely that psychiatric symptoms alone can fully explain the cognitive deficits.

A more likely scenario is that both psychiatric symptoms and cognitive impairments are part of the neuropsychiatric syndrome found in patients with chronic HCV infection[15].

Fatigue

Chronic fatigue is the most common symptom of chronic HCV infection, with a prevalence ranging from 20% to 80% in different cohorts[2].

It is perceived as a feeling of physical and mental exhaustion that is often accompanied by muscle and joint pain, sleep disturbances and restless legs syndrome[4]. This "central" fatigue differs from the "peripheral" fatigue linked to muscle weakness.

Fatigue is influenced by psychological, social and physical factors, so a direct virus effect on this symptom remains unclear[2]. In fact, it is the hallmark of depression and consequent apathy, demotivation, unrefreshing sleep, disinterest and loss of energy [42].

Moreover, a positive association between fatigue and hepatic fibrosis has been demonstrated by Teuber *et al*[43] In fact, the degree of fatigue assessed with the Fatigue impact scale was higher in patients with severe fibrosis or cirrhosis than in patients with mild fibrosis, mostly in the physical domain.

However, central fatigue and depression may share possible biological changes in the brain.

It has been hypothesized that alterations in cerebral metabolism and neurotransmission in the limbic system, ascending reticular system, globus pallidus and putamen are associated with chronic fatigue[4].

Dopamine reduction in the limbic system and ascending reticular system could be secondary to cytokine-induced reduction of tetrahydrobiopterin, an enzyme involved in dopamine synthesis[44,45]. In fact, a study showed an association between fatigue and peripheral inflammation, suggesting that activity of the peripheral immune system may contribute to this symptom[46].

Impairment of serotonin transmission, implicated in depression, has also been correlated with fatigue. This finding is confirmed by the fact that treatment with ondansetron, a competitive antagonist of the serotonin receptor, is effective in improving fatigue. Therefore, this evidence supports the role of serotonin pattern dysfunction in causing fatigue and is in line with data showing a decrease in serum tryptophan levels and a reduction in serotonin synthesis in patients with chronic HCV infection[17,47].

Depression

A high frequency of anxiety and depression has been reported in patients with HCV infection and these psychiatric pathologies negatively impact the quality of life[2].

The prevalence of HCV infection in patients with psychiatric disorders is significantly higher than in the general population and can affect 20% to 50% of infected patients compared to 10% of healthy subjects[2,6]. This association would be greater in genotype 3a[48,49].

An important aspect is that depression can affect patient prognosis as it is associated with poor adherence to antiviral therapy[50]. Therefore, multidisciplinary involvement is essential both to ensure adherence to treatment and for management of depressive symptoms[45].

Depression can be reactive to physical and psychosocial sequelae of HCV infection. Being aware of infection itself constitutes a risk factor for depression due to the impairment of family and intimate relationships, stigmatization and social marginalization[2,45]. Frequent coexistence of intravenous substance abuse[51] or symptoms such as fatigue and cognitive decline may also explain the onset of depression[52]. Depression can also be secondary to comorbidities associated with HCV infection such as diabetes mellitus and insulin resistance, metabolic syndrome, cardiovascular disease and inflammatory conditions such as cryoglobulinemia and arthritis which cause skin manifestations, motor and sensory neuropathies, joint pain and reduced joint mobility[45].

A higher burden of physical symptoms from HCV is associated with increased depression, and this burden increases with complications of cirrhosis such as ascites, sarcopenia and encephalopathy, causing limitations in physical and social function [44].

However, depression is not entirely attributable to these factors.

Endotoxins and peripheral cytokines, in particular IL-2, IL-6 and IL-10, act on the blood-brain barrier stimulating prostaglandin E2 release in the central nervous system. This in turn stimulates the release of additional cytokines by microglia cells, such as IL-1, IL-4, IL-6, IL-12, IL-18 and TNF-α. These mediators can inhibit neurotransmission in areas involved in affective responses and emotional processes[53], and induce the activity of enzymes that metabolize tryptophan into neurotoxic metabolites rather than serotonin[54]. In turn, serotonin deficiency can cause an excessive release of inhibitory neurotransmitters leading to depressive symptoms. In fact, serotonergic projections from midbrain nuclei modulate the activities of cortical and subcortical structures involved in behavior, mood and attention regulation[16].

The cytokines can excessively stimulate the hypothalamus-pituitary-adrenal system leading to excessive endogenous cortisol production which can alter hippocampus neurotransmission. In addition, corticotropin-releasing hormone can directly cause neurotoxicity[45].

Confirming the direct role of virus in causing depression, in a cross-sectional study of patients with liver disease of different etiology [hepatitis B virus (HBV), HCV, alcohol abuse and metabolic syndrome], only the presence of HCV was found to be independently associated with depression. This suggests an additional and causal mechanism due to virus presence that contributes to depression.

Quality of life

The World Health Organization defines the quality of life as the individual's perception of their state of physical, mental and social well-being[2].

Several studies have highlighted the impact of chronic HCV infection on quality of life, even without significant liver disease and regardless of modality of virus acquisition, for example previous substance abuse[2].

Even in the absence of debilitating symptoms, HCV compromises quality of life by negatively impacting the physical and mental well-being of patients. Nonspecific symptoms such as asthenia, irritability, general malaise and musculoskeletal pain can occur in patients with chronic HCV infection and in many cases reduce the sense of physical well-being causing emotional problems[1]. Indeed, in quality of life studies in which the SF-36 questionnaire was used, depression and fatigue were found to be independent predictors of reduced quality of life[45].

Other factors involved in the deterioration of quality of life are the psychological impact of prognosis, comorbidities associated with infection and social stigmatization, the shame of being infected and the fear of infecting other people, unemployment and financial problems.

Furthermore, until a few years ago, patients were concerned about the limited eradication capacity of interferon-based regimens and about the disabling adverse effects associated with this therapy[1].

Quality of life is more compromised in patients with HCV than HBV infection and this may suggest a direct role of HCV virus on this aspect[55]. In fact, several prospective studies have shown that infected patients compared to non-infected, have lower scores related to quality of life even before knowing their serological status[56] and that antiviral therapy and viral suppression induce an important clinical improvement[1].

Therefore, HCV infection has a direct effect on quality of life that can be aggravated by psychological factors related to diagnosis and clinical complications of infection itself[2].

Several studies have identified which factors are independently associated with reduced quality of life as they can potentially aggravate the burden of infection.

A study by Jang *et al*[57] showed that the SF-36 physical and mental component summary scores of HCV patients, were below the normal limits. However, those with decompensated cirrhosis had the lowest health-related quality of life (HRQoL), while HCC and chronic hepatitis patients had similar HRQoL results.

Confirming this hypothesis, Golabi and colleagues demonstrated that severity of liver disease may effects patients' HRQoL; in fact worsening hepatic dysfunction in patients with cirrhosis (higher MELD score) and development of complications, such as ascites and hepatic encephalopathy, can cause severe impairment of HRQoL[58].

Thus, there may be a stage-dependent relationship between the degree of fibrosis and the impairment of QoL in patients with severe fibrosis or cirrhosis, compared to those with moderate fibrosis.

HCV-ASSOCIATED NEUROCOGNITIVE DISORDER AND MINIMAL HEPATIC ENCEPHALOPATHY

There is increasing evidence to support that cognitive impairments in patients with chronic HCV infection occur before the development of cirrhosis and are independent of liver function, viral load and genotype[15].

Many factors that may coexist in infected patients can aggravate cognitive decline such as the presence of liver cirrhosis and minimal hepatic encephalopathy (MHE), drug abuse and psychiatric disorders such as anxiety and depression[2]. On the other hand, pre-infectious intellectual functions and educational level can be protective against cognitive decline[59,60].

Genetic factors have also been seen to play a role in the development of cognitive disorders; in particular, some allelic variants of *ApoE* gene result in a lower impairment of attention and executive functions[61].

The term HCV-associated neurocognitive disorder (HCV-AND) is used to refer to this spectrum of alterations unrelated to advanced liver disease and therefore distinct from potentially reversible complications seen in patients with MHE[16].

Patients with chronic HCV infection may present symptoms related to cognitive decline. In particular, the areas mainly involved are: Executive functions: involved in problem solving, monitoring one's own behavior, self-control, cognitive flexibility, working memory, volition, sustained attention and logical reasoning; verbal learning and verbal recall. Therefore, in HCV patients there is a prevalent involvement of the frontal lobe, which is responsible for alterations of executive functions.

Cognitive domains related to posterior brain regions, primarily involved in visuospatial and visual perceptual abilities and constructive practice, are rarely involved; conversely these regions are mainly altered in patients with MHE[16]. Thus, there are some differences from both a pathogenic and a clinical-diagnostic point of view between HCV infected patients and those with hepatic encephalopathy.

Type C hepatic encephalopathy is one of the complications seen in patients with liver cirrhosis of different etiology and is a consequence of severe hepatocellular damage or porto-systemic shunts. This condition can manifest itself with a wide spectrum of neurological and psychiatric symptoms whose spectrum varies from subclinical forms to more severe forms characterized by a complete alteration of the state of consciousness.

To date, hepatic encephalopathy is divided into two types: covert which includes minimal hepatic encephalopathy and grade I hepatic encephalopathy, and overt (OHE), which includes grade II, III and IV hepatic encephalopathy[62-64].

OHE is characterized by personality alterations such as apathy, disinhibition and irritability, alterations in the sleep-wake cycle with excessive daytime rest, disorientation in time and space, altered state of consciousness, asterixis, acute confusion, stupor and finally coma.

The pathogenesis shown by hematoxylin and eosin staining is complex and sees a central role of ammonia which determines the activation of oxidative stress in astrocytes and swelling due to intracellular accumulation of osmotically active substances such as lactates and glutamine; a contribution is also linked to metal accumulation and neurosteroids that are able to activate inhibitory GABA-A receptors [65]. However, HE pathogenesis could be considered multifactorial. In fact, systemic inflammation and metabolites other than ammonia are involved.

MHE is the mildest form of hepatic encephalopathy, characterized by poor performance on psychometric tests that assess attention, working memory, psychomotor speed and visuospatial skills. Despite being subclinical, minimal HE has a heavy burden both on patients and caregivers. In fact, the relationship between MHE and QoL, driving capacity and sleeping is well known. Therefore, it is important to detect MHE, which can be recognized in asymptomatic patients using appropriate psychometric tests and neurophysiological methods.

MHE is a frequent complication of liver cirrhosis, affecting up to 80% of patients and, despite its minimal clinical expression, constitutes a significant problem as it is associated with burden on the caregiver, inability to drive, sleep disturbances, falls, worsening quality of life and risk of developing OHE[60].

Cognitive decline in patients with MHE mainly affects the following domains: Selective attention and vigilance; Psychomotor speed, with lengthening of reaction times; Inhibitory responses, with loss of inhibitory control; Visuospatial skills; Verbal skills are relatively preserved[66].

DIAGNOSTIC STRATEGIES IN HCV-AND

To distinguish between HCV-AND and MHE in terms of cognitive decline, a multimodal approach is required that includes assessment of liver function, brain imaging and extensive neuropsychological tests that assess broad aspects of cognition and not just those related to frontal executive functions[16].

In patients with suspected HCV-AND, tests that can be used to analyze the various cognitive domains are: Verbal learning and episodic memory: Rey auditory verbal learning test, Hopkins verbal learning test total recall, brief visual memory test revised total recall, digit forward span, verbal learning and memory test; Working memory: digit backward span, letter number sequencing, paced auditory serial addition task; Visuospatial skills and planning: Rey-Osterrieth complex figure test (ROCF) in the immediate trial; Lexical skills and executive functions: phonemic and the semantic verbal fluency tests, color-word-interference test (Stroop), trail making test part B,

Wisconsin card sorting test, ROCF in the 10-min delayed recall trial; Logical thinking: verbal judgment test; Non-verbal intelligence: Horn's performance test system[67-69].

Table 1 summarizes the main tests and their relative characteristics that can be used to investigate the altered cognitive domains in patients with HCV-AND.

Neuropsychological tests, such as potential P300 related to the event, have shown an increase in latency and a reduction in peak amplitude in patients with cognitive decline, regardless of confounding factors such as fatigue and depression[4].

Diagnostic strategies in MHE

The optimal measure for diagnosing MHE is still debated. In fact, none of the methods proposed cover the complexity and the heterogeneity characteristic of this syndrome. Diagnosis of MHE can be made with psychometric tests and electrophysiological tests. Tests used to investigate domains known to be affected in patients with MHE are: Selective attention and vigilance: critical flicker frequency, continuous reaction time test, SCAN test; Psychomotor speed and visuospatial skills: psychometric hepatic encephalopathy score, Stroop test, repeatable battery for the assessment of neuropsychological status; inhibitory responses: inhibitory control test; animal naming test.

Similar to non-cirrhotic patients with chronic hepatitis C, patients with MHE present emotional alterations such as depression, anxiety and alexithymia[70], especially in the final stages of their pathology which is characterized by medical complications, hospitalizations, functional limitations and changes in body image[1].

They also have a reduced quality of life which can be linked to various mechanisms such as physical problems and complications related to liver cirrhosis and psychiatric symptoms. In fact, depression, anxiety and alexithymia are among the major determinants of the quality of life of cirrhotic patients, regardless of the severity of the liver disease.

The presence of these disorders can be explored using tests such as: Depression: Zung-self depression rating scale; Anxiety: Spielberger state-trait anxiety inventory Y1 and Y2; Alexithymia: Toronto alexithymia scale[70].

Imaging in HCV-AND

Over time, several imaging studies have been conducted with the aim of finding a biological relationship between HCV infection and cognitive decline[2].

On magnetic resonance imaging, patients with cognitive decline show an increase in signal intensity in T2-weighted sequences in peri-ventricular white matter[71]. Furthermore, atrophy and cytoarchitectural alterations are observed in the lobe of insula which is involved in attention[16].

SPECT studies have demonstrated an alteration in mesencephalic/hypothalamic and striatal dopaminergic transporters in up to 60% of patients and this finding is associated with executive-attentional function decline[18].

On the other hand, a positron emission tomography (PET) with 18-fludeoxyglucose (18-FDG) study, demonstrated hypometabolism in the anterior cingulate gyrus and in the upper and middle frontal gyrus, responsible for fatigue and impaired attention, and in the parahippocampal gyrus and caudate nucleus bilaterally, causing anxiety, depression and lengthening of reaction times[16]. These data confirm the role of impaired dopaminergic transmission in the cognitive decline of patients with chronic HCV infection[2,72].

Spectroscopy studies have instead highlighted an increase in the choline/creatine ratio in the basal ganglia and frontal white matter[73], which is associated with executive functions decline[6]; another study showed a reduction in the N-acetyl aspartate/creatine ratio in frontal grey matter, which proves a reduction in the neuronal population in this area. These findings suggest an increase in cell membrane turnover and a decrease in neuronal functions, respectively[74].

The increase in myo-inositol levels agrees with chronic cellular inflammation induced by virus[75] and has been associated with fatigue and cognitive decline[76].

Overall, neurophysiological and imaging tests are important tools for characterizing cognitive impairments as they can anticipate dysfunction before it manifests clinically. They are also not subject to bias due to test batteries, self-reported measurements, different socio-cultural levels and different degrees of school education[15].

Imaging in HE

Imaging findings in patients with liver cirrhosis are different to those previously described for patients with HCV-AND.

In fact, spectroscopy studies confirm that the pathogenetic mechanism for these symptoms is different to that observed in patients with chronic hepatitis C. In

Table 1 Tests exploring cognitive domains[67-69]

Test	Tested domains	Description
Rey auditory verbal learning test (RAVLT)	Immediate memory and learning	After hearing a list of 15 unrelated words, the patient has to recite as many words as he can recall for a total of five trials.
Hopkins verbal learning test total recall (HVLT)	Verbal memory	This test consists of three parts: total recall, delayed recall and recognition. The test consists firstly of three free recall learning trials with 12 words from three different categories such as professions, food and sports (HVLT total recall). 20-min later, the patient has to name the words from the free recall learning list (HVLT delayed recall). Finally, the yes/no recognition trial is completed (HVLT recognition).
Brief visual memory test revised total recall (BVMT)	Visuospatial memory	In three learning trials, the patient views the stimulus page for 10 s and then has to draw as many figures as possible in their correct location. A delayed recall trial is administered after a 25-min delay. Lastly, a recognition trial, in which the patient has to identify which of the 12 figures were included among the original geometric figures, is administered.
Digit forward span and digit backward	Verbal learning and episodic memory	The Digit Span consists of two different tests: digits forward (repeating digits forward) and digits backward (repeating digits in reverse). This test consists of pairs of sequences of numbers read by the examiner. When the patient repeats correctly the first sequence, the examiner reads the next one, which is longer by one number than the previous one, and continues until the patient fails a pair of sequences or correctly repeats the last sequence consisting of nine numbers.
Letter number sequencing (LNS)	Working memory	During administration, the patient listens to a series of numbers and letters read by the examiner. Then he has to repeat the numbers in ascending order and letters in alphabetic order.
Paced auditory serial addition task (PASAT)	Auditory-verbal divided attention; selective and sustained attention	Numbers are presented orally at a rate of about 1-2 s. The patient has to add pairs of numbers, adding each number to the previous one. In this way, the first number will be added with the second, the second with the third, the third with the fourth and so on.
Rey-Osterrieth complex figure test in the immediate trial (ROCF)	Visoconstructive ability, problem solving, perceptual organization and visual memory	There are 18 elements overall. In the first part, the patient has to copy a figure (ROCF copy). Three minutes later, he has to perform the figure again without the stimulus (ROCF immediate recall). Two points are given when the elements are correctly reproduced; one point when the production is distorted, incomplete but placed properly, or completed but placed poorly; half point when the elements are distorted or incomplete and placed poorly; zero point when the elements are absent or not recognizable.
Phonemic and semantic verbal fluency test	Lexical skills and executive functions; verbal functioning	For the phonemic part, the patient has to list as many words as possible starting with an F, A or S during 60-s intervals. For the semantic part, he has to list as many animals as he can during a 60-s interval.
Color word interference test (Stroop)	Lexical skills, cognitive flexibility, selective attention and response inhibition (or disinhibition)	Patient has to read three different tables as fast as possible. Two of them represent the "congruous condition" in which he has to read names of colors printed in black. Conversely, in the third table, color-words are printed in an inconsistent colour ink. In this incongruent condition, the patient has to name the ink colour instead of reading the word.
Trial making test part B (TMT-B)	Lexical skills, executive functions, cognitive flexibility	In the TMT-B, the patient has to draw lines sequentially connecting numbers and words alternately. The score represents the amount of time required to complete the task.
Wisconsin card sorting test (WCST)	Lexical skills and executive functions; cognitive reasoning	Patient has to classify cards according to different criteria. There are different ways to classify each card and the only feedback is whether the classification is correct or not. Classification possibilities are the color of symbols, the shape of symbols and the number of shapes on each card. The classification rule changes every 10 cards; so, once the patient has figured out the rule, he will start making mistakes when the rule changes. This test measures how well the patient is able to adapt to changing rules.
Verbal judgment test (VJT)	Logical thinking	This test consists of an oral interview. The examiner asks the patient how he would solve a hypothetical situation.
Horn's performance test system (LPS)	Non-verbal intelligence	This test comprises 40 rows, each with 9 numbers and/or letters. Every row has a logical order with one letter or number that does not fit and the goal is to identify the mismatched character. The outcome parameter is the sum of correct rows.

RAVLT: Rey auditory verbal learning test; HVLT: Hopkins verbal learning test total recall; BVMT: Brief visual memory test revised total recall; LNS: Letter number sequencing; PASAT: Paced auditory serial addition task; ROCF: Rey-Osterrieth complex figure test in the immediate trial; TMT-B: Trial making test part B; WCST: Wisconsin card sorting test; VJT: Verbal judgment test; LPS: Horn's performance test system.

particular, the prevailing pattern is characterized by a reduction of the choline/creatine and myo-inositol/creatine ratio and an increase in the glutamine/creatine ratio.

The exact mechanism underlying the reduction in choline levels has not been fully defined.

This is partly explained by a decrease in choline transport, malnutrition and cerebral metabolism alteration secondary to liver dysfunction.

Glutamine increase could be a consequence of hyperammonemia. In this condition, astrocytes detoxify ammonium converting it into glutamine which causes low-grade cerebral edema and symptoms of HE due to its osmotic effect[77].

In addition, brain MRI reveals basal ganglia hyperintensity in T1-weighted sequences, most likely related to manganese deposits[77].

EFFECTS OF ANTIVIRAL THERAPY

Until recently, INF and pegylated INF have been the only effective treatments for HCV infection as modulators of the immune response, showing a sustained virological response (SVR) of up to 50%-80% when combined with ribavirin.

This therapy is associated with a high prevalence of neuropsychiatric disorders, which affect up to 70% of subjects. Predominant symptoms are mood disorders such as anxiety and depression, psychosis and manic agitation, suicidal ideation and cognitive decline. These disorders disappear at the end of therapy[16] and can be attributed to numerous pathophysiological complications associated with treatment such as alteration of monoamine metabolism, increase in apoptosis, reduction in cerebral neurotrophic factor, alteration of hypothalamic-pituitary-adrenal axis[78] and alteration of frontal-subcortical circuits[15]. Co-factors for the development of this disorder during antiviral therapy are low education, a known history of psychiatric disease and high serum concentrations of IL-6[79]. In subjects infected with HCV and treated with INF, PET studies with 18-FDG have shown a reduction of metabolic activity in the prefrontal cortex which agrees with a depressive state[80].

HCV infection treatment has been radically changed since the introduction of direct-acting antivirals (DAAs). INF-free regimens are better tolerated and are highly effective, reaching an SVR greater than 95%; furthermore, the simplicity of dosage and their safety profile guarantee good compliance.

These antiviral agents considerably improve extrahepatic symptoms underlining the direct and indirect pathogenetic role of virus in precipitating these disorders. Obviously, the success of this strategy depends on achievement of the SVR[6]. This has been confirmed in several clinical studies.

A recent study by Nardelli *et al*[1] showed an improvement in all domains investigated using the SF-36 questionnaire after therapy with DAAs, but also in psychiatric symptoms such as anxiety, depression and alexithymia, which were found to be among the major determinants of altered quality of life.

Some trials have shown that the improvement in quality of life is evident during the first 4 wk of therapy, coinciding with the suppression of viral replication, then continuing until the end of the therapy and beyond during the follow-up[81].

Patients who achieve SVR 12 wk after sofosbuvir therapy, including those with cirrhosis, have reported significant improvements in quality of life, general well-being, fatigue, sleep disturbances, emotional state and physical functioning[16,81].

When INF-based regimens are used, quality of life worsens during treatment due to poor tolerability of these regimens and then improves in those that reach SVR at the end of treatment. Similarly, ribavirin-based regimens are associated with greater impairment of quality of life during treatment than ribavirin-free regimens[81].

However, therapy with new antivirals improves not only psychiatric symptoms, but also cognitive changes.

The study by Kleefeld *et al*[68] demonstrated that after DAA therapy, fatigue and mental health, as well as executive functions, processing speed, learning, verbal fluency and motor skills improved in both monoinfected and those with HIV infection. However, no differences were found in working memory and verbal memory before and after therapy. The hypothesis that has been advanced to explain this phenomenon is that the compartmentalization of virus in the central nervous system may represent a potential source of reactivation of virus, thus explaining the persistence of some cognitive symptoms at the end of therapy with DAAs[45,66]. Therefore, it is unclear to what extent the new antiviral regimens can guarantee a return to normal[81].

To confirm this fact, neurocognitive tests and spectroscopy images were compared in several studies before and after antiviral therapy; patients who reached SVR had a significant reduction in the choline/creatine and myo-inositol/creatine ratio as well as an increase in cognitive functions compared to untreated patients or patients who did not achieve SVR[82].

Mixed cryoglobulinemia is strongly associated with HCV infection and its clinical expression varies from completely asymptomatic to severe vasculitis that manifests with dermatological, renal, neurological and musculoskeletal complications. Many authors report that up to 30% of HCV-infected patients have mixed cryoglobulinemia, but of these less than 10% develop symptoms. Symptomatic patients usually have higher immunoglobulin values.

Mortality and morbidity associated with mixed cryoglobulinemia are significant and treatments are focused on either eradicating the viral trigger with antiviral therapy or inhibiting B cell proliferation with immunosuppressive therapy, usually with steroids and rituximab in severe forms.

INF and ribavirin therapy has been associated with clinical and immunological improvement in patients with mixed cryoglobulinemia, in terms of cryoglobulin, IgM, rheumatoid factor and complement levels. Therefore, in patients who reach SVR, INF-based therapies exert a beneficial effect in those with HCV-CV (62%-78%). However, patients with HCV-CV have a lower rate of achievement of SVR than patients who do not (48.6% *vs* 61.4%). In fact, these patients tend to have comorbidities that can affect the achievement of SVR.

Almost all studies have also shown a complete or partial clinical response in patients with mixed cryoglobulinemia who reach SVR after DAAs therapy (61%-100%). The highest clinical response was seen in the study by Saadoun *et al*[83] in which all patients achieved a clinical response (90% complete and 10% partial) after 12 wk or 24 wk of sofosbuvir/daclatasvir therapy. A similar result was obtained by Gragnani *et al*[84] with complete or partial clinical response achieved in 93% of patients.

However, therapeutic effect may differ depending on the type of organ involved. The study by Gragnani *et al*[84] showed that the effect on dermatological symptoms is greater than that on nephrological and neurological symptoms. Furthermore, palpable purpura, renal pathology and skin ulcers resolve more rapidly than sicca syndrome, fatigue and peripheral neuropathy. In fact, neurological symptoms and sicca syndrome can be irreversible if there is permanent organ damage.

Among patients who have been cured of the infection and who continue to present symptoms of cryoglobulinemic vasculitis, an alternative explanation should be considered such as B-cell non-Hodgkin lymphoma. In fact, B lymphocyte proliferation can reach an autonomous and virus-independent replication phase demonstrated by the persistence of B lymphocyte clones t (14; 18) and small quantities of HCV-RNA in the lymphatic system after eradication therapy[85].

CONCLUSION

HCV can strongly impair the neuropsychological panorama of cirrhotic patients. In this review, we summarized the most innovative aspects of central nervous system impairment in HCV positive patients. Particular attention was paid to patients with advanced liver disease and minimal HE. DAAs therapy completely revolutionized the standard of care for HCV infected patients. In fact, these drugs, characterized by great efficacy and tolerability, have made it possible to reach levels of SVR unthinkable in the twentieth century. Further studies are needed to evaluate in depth the effects of HCV eradication, even in the long-term, on the neuropsychological alterations of these patients, and to determine if there is a residual deficit, which cannot be resolved with viral eradication.

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MINIREVIEWS

Muscular abnormalities in liver cirrhosis

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Abstract

Sarcopenia is becoming a well-established player in evaluating patients with chronic liver disease. Data regarding its clinical significance and consequences in the course of liver disease have been growing; many of the data support the idea that it impacts decompensation event frequency, prolonged hospitalization, and mortality, as well as providing the possibility to better prioritize patients on lists awaiting liver transplantation. When assessing the whole clinical scope of the field, which includes malnutrition and frailty, as well as the complete spectrum of muscle mass, strength, and function, it becomes clear that a well-founded approach in everyday clinical practice is essential. In this respect, this article attempts to unveil the most recently published data regarding possible methods and modalities that could be used to diagnose sarcopenia as early as possible, along with the required accuracy and reliability. From the most important field discoveries to data that need further clarification, the merits and weaknesses of the very diverse existing evaluation methods are presented. Finally, a critical overview is given, in an attempt to discern study lines of importance from those that could pose further ambiguity for the theme. The author also poses relevant questions that remain unanswered but are of clinical importance in the field.

Key Words: Sarcopenia; Liver cirrhosis; Frailty; Muscle strength; Nutrition indices; Risk factors

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Core Tip: Knowledge regarding the influence of sarcopenia in the course of chronic liver disease has greatly expanded in the past ten years, especially with respect to cirrhosis. Data show that it has a great influence on disease decompensation and patient mortality, providing clues for the development of newer evaluation modalities and sarcopenia indices. Nonetheless, data regarding the therapeutic consequences and interventions remain scarce. This article attempts to summarize the current state of

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knowledge of this important clinical topic with a critical evaluation of some related groundbreaking studies.

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INTRODUCTION

Sarcopenia is an important part of the medical evaluation and treatment for chronic liver disease, especially in the advanced stage of the disease (*i.e.*, cirrhosis), not only due to the very frequent coexistence of both medical entities but also because of its impact on the related clinical outcomes. This article focuses on the clinical aspects and consequences of sarcopenia in liver cirrhosis, common diagnostic procedures, and suggestions for clinical therapy. A review of the presented field in the scientific literature is provided, with the intentional omission of pathophysiology, for better transparency, clinical applicability, and an attempt to critically evaluate the most recent study findings.

To facilitate the understanding of common medical terms, a short paragraph summarizing their definitions is presented (see also Figure 1), followed by a brief description of the pathophysiological processes involved. For a more detailed review of the pathophysiology of sarcopenia in liver cirrhosis-especially regarding the possible influence of the gastrointestinal microbiota on the occurrence and course of muscle dysfunction-the author recommends some excellent reviews in this field[1-4].

DEFINITIONS

Malnutrition is defined as a measurable change in physical and mental functions secondary to altered body composition and cell mass, resulting in an impaired quality of life and poor clinical outcomes. It is the consequence of insufficient protein and energy supplies.

It is well admitted that malnutrition participates in the onset of sarcopenia but the link between these two nutritional concepts remains confusing[5].

Sarcopenia is defined as the loss of muscle mass, muscle strength, and reduced physical function.

Frailty is a condition of increased vulnerability to endogenous and/or exogenous stressors associated with physiological decline.

Myosteatosis is pathological fat accumulation in muscles.

PATHOPHYSIOLOGY

Sarcopenia is often equated as a state of advanced malnutrition. The condition is associated with complications such as poor mobility and quality of life and increased mortality. Muscle loss in sarcopenia is not solely at the expense of muscle atrophy, it also involves replacing muscle cells with fat and connective tissue. Protein supply in a sarcopenic patient is low due to chronic muscle degradation which is most clearly manifested during metabolic stress when muscle proteins should be mobilized very quickly to provide the body with amino acids for the liver, gut and immune system function. Sarcopenia in patients with liver cirrhosis is connected to age-related decline in muscle mass and to malnutrition. Studies show that malnutrition develops due to negative energy balance, loss of appetite, rapid satiety and poor food absorption. Concurrent illnesses or addictions (bacterial growth in the gut, pancreatic exocrine insufficiency, alcoholism) exacerbate the condition. Iatrogenic losses include ascites paracentesis and use of laxatives and diuretics. Frequent endoscopic imaging and laboratory examinations also affect diet and weight loss as well as sarcopenia. Therefore, liver cirrhosis is a state of accelerated starvation. In liver cirrhosis, cytokines

Malnutrition - measurable change in physical and mental funcons secondary to altered body composion and cell mass, resulng in an impaired quality of life and poor clinical outcomes

Sarcopenia - loss of muscle mass, muscle strength and reduced physical funcon

Frailty - condion of increased vulnerability to endogenous and/or exogenous stressors associated with physiological decline

Figure 1 Main definitions of the presented topic.

that stimulate catabolic processes in the body are released. Due to the low glycogen stores, energy production is directed towards the breakdown of fatty acids. Gluconeogenesis is mainly pursued through amino acids breakdown, which worsens protein loss. Advancement of the state is also exacerbated by growth hormone and testosterone deficiency, decreased physical activity, and more frequent occurrence of hepatocellular carcinoma (HCC). Patients with liver cirrhosis are prone to replacing muscle fibers with fat cells (myosteatosis), which may further accelerate loss of muscle function and decline in muscle strength. Connection between hepatic encephalopathy (HE) and sarcopenia is in a form of a vicious cycle where ammonia is likely to be directly responsible for mitochondrial oxidative damage and autophagy of skeletal muscle cells; it also inhibits protein synthesis in skeletal muscle through stimulation of myostatin[1-4].

CLINICAL SIGNIFICANCE AND CONSEQUENCES OF SARCOPENIA

To facilitate the clinical application and evaluation of sarcopenia tests, The European Working Group on Sarcopenia and Older People provided consensus criteria for the diagnosis of sarcopenia using muscle mass, muscle strength, and muscle function as a practical clinical definition. In 2019, a revised definition was published. Pre-sarcopenia is defined as the presence of low muscle strength without its impact on muscle mass/quality or muscle function. Sarcopenia is defined as a low muscle strength with additional low muscle mass or quality. For severe sarcopenia, muscle mass, muscle strength, and muscle function needed to be impaired. As can be seen from the definition, muscle mass does not condition muscle function or strength. The end result of the sarcopenia process is a decline in muscle functional abilities assessed by frailty [6].

To assess frailty, the 5 Fried's phenotypes of frailty as cited by Sinclair[7] are used. While patients with 3 or more phenotypes are defined as frail, patients with 1 or 2 phenotypes are defined as prefrail, and those with no phenotype as robust[7]. The concomitant presence of sarcopenia and obesity is significantly associated with prefrailty and frailty, especially in women with cirrhosis[8]. According to a well-defined description by Buchard *et al*[5], "sarcopenia [is] not identical to frailty but there is a major overlap between definitions and diagnosis criteria of the two phenotypes. Frailty is a more multidimensional concept encompassing not only muscle conditions but also exhaustion, well-being, disability, dependency, and cognitive state. Loss of skeletal muscle mass and function remains a strong substratum of frailty, as in malnutrition. The absence of sarcopenia certainly does not rule out frailty but clues of frailty must lead to a complete body composition evaluation".

In a well-designed study, a group of authors led by Traub *et al*[8] evaluated the applicability of the latest definition of sarcopenia in patients with cirrhosis. Computed tomography (CT) examination was used to assess muscle mass, hand grip test to assess muscle strength, and 4 m gait speed test to assess muscle function. Compared to the 2019 definition, they observed that the 2010 definition identified more sarcopenia cases in male patients. In patients with cirrhosis, muscle strength seems to be preserved longer, while muscle mass is already reduced, leading to a significant difference in sarcopenia diagnosis rates when using the 2019 definition. However, the gender imbalance seen in the 2010 definition seemed to be less pronounced with the 2019 definition. They objected to the lack of data whether the 2010 or 2019 criteria is better to predict clinical complications, poorer prognoses, and the effect of specific interventions[8].

Sarcopenia is present in 30% to 70% of patients with cirrhosis, probably less frequently in the population of patients with metabolic liver disease. The prevalence increases with the disease stage and increases sharply before liver transplantation[7,

9]. Based on the Child–Pugh score, the annual rate of decrease in skeletal muscle mass is 1.3% in Child–Pugh A patients, 3.5% in Child–Pugh B patients, and 6.1% in Child–Pugh C patients[10]. The risk of developing sarcopenia is associated with male gender, ascites, and the degree of renal and hepatic dysfunction[9]. Overweight and obesity are as frequent as in general population, ranging from 20% to 40% and aggravating prognosis both in compensated and in decompensated cirrhosis[11]. Sarcopenic obesity (coexistence of sarcopenia and obesity) is present in one-fifth to one-third of patients with cirrhosis. It is determined by using an assessment of sarcopenia to which an assessment of obesity using a body mass index (BMI ≥ 25 or ≥30 kg/m^2) is added. BMI corrected for ascites is probably the most practical to use (see below under the Anthropometry section), although it may shift patients to a lower BMI grade[12]. Myosteatosis occurs in up to 50% of patients with cirrhosis and can be identified in both sarcopenic and non-sarcopenic patients with or without obesity. It meditates inflammatory responses and has been associated with lower muscle function and strength, muscle atrophy, and physical disabilities[12]. It affects mortality, more through loss of muscle function than through liver cirrhosis determinants. It probably plays an important role in the occurrence of HE[13]. Microscopic analysis of skeletal muscle fibers references special attention to atrophy of fast-twitch fibers and reduction in muscle fiber size. Reduced skeletal muscle fiber size and shift toward wider fast-twitch fibers (increased proportion of type IIA/IIX fibers concurrent with lower proportions of type IIA fibers) were visible in the muscle of a sarcopenic patient [3].

The main clinical consequences of sarcopenia are increased mortality *per se*, increased mortality from systemic bacterial infection, the occurrence of HE after transjugular intrahepatic portosystemic shunt (TIPSS) insertion, and the more frequent occurrence of acute on chronic liver failure. Regardless of the underlying disease, it has a significant effect on mortality in patients with HCC[9]. Ebadi *et al*[3] point out that it would be important to define strategies maintaining muscle mass in candidates prior to the TIPSS insertion. A study by Al-Azzawi *et al*[14] confirmed that the existence of sarcopenia in alcoholic hepatitis prolongs hospital treatment. In the case of sarcopenia, patients had a more severe course of the disease. To date, there is no solid evidence linking portal hypertension to known sarcopenia indices. In a well-defined study, hepatic venous pressure gradient (HVPG) was shown to inversely correlate with adipose tissue indices, while CT sarcopenia markers were unrelated to the degree of portal hypertension[15]. On the other hand, the Austrian study group demonstrated poorer survival and an increased risk of further cirrhosis decompensation in the group of patients with HVPG > 20 mmHg by comparing CT sarcopenia indices and invasively assessed clinically significant portal hypertension (CSPH)[16].

In the group of patients awaiting liver transplantation, sarcopenia can affect relegation from the waiting list due to excessive perioperative risk, increased need for hospitalization, and prolonged hospitalization with multiple complications after organ transplantation[9]. Decline of muscle function has a significant impact on mortality and the occurrence of complications requiring hospital treatment[17]. The presence of sarcopenic obesity in this group of patients is relatively common and is associated with higher mortality than if the entities were present separately[11]. After organ transplantation, there is a lack of clinical data regarding the impact of sarcopenia on long-term clinical outcomes[13].

Given the above-mentioned situation, it is not surprising that there are calls for the inclusion of sarcopenia among the factors of transplantation priority [Model for End-Stage Liver Disease (MELD) – score *vs* sarcopenia-MELD score]. A thorough study in this area evaluated patients on a transplant waiting list. To define sarcopenia, CT measurements with proposed cut-off values were used (see below). In this cohort of European patients, they demonstrated the sarcopenia impact on the increased mortality. When comparing MELD to sarcopenia-MELD score, the authors also confirmed a much lower priority of patients with MELD < 15. It has been observed that a better scoring option would include one encompassing the patient's age and previous occurrence of HE[18]. In the literature, other authors support the idea for better transplant priority of patients with sarcopenia and low MELD score to reduce the risk of waiting list mortality and to improve the overall outcome[19]. They also point to the strong impact of sarcopenia reflected in the mere fact that it is equivalent to adding 10 points to the MELD score if presented[20].

From a clinical point of view, it is important whether sarcopenia is worth assessing in the group of patients with cirrhosis and HCC. Sarcopenia probably affects the recurrence or progression of HCC in patients who are candidates for liver transplantation. Presumably, sarcopenia in this group of patients affects long-term mortality after surgical treatment of a single lesion with partial hepatectomy. While sarcopenic

patients have a smaller volume of preserved liver tissue, there is no data connecting sarcopenia and postoperative decompensation in this group. There is also almost no data relating the effect of sarcopenia in HCC treatment with radiofrequency ablation. In the group of patients receiving transarterial chemoembolization, sarcopenia affected survival and response to treatment. In the group of patients receiving chemotherapy with sorafenib, sarcopenia affected disease progression. Numerous data suggest that sorafenib itself has a significant effect on reducing muscle mass. The condition is codependent in both directions as sarcopenia has an impact on the systemic treatment side effects severity (*e.g.*, enteritis), and thus on the sorafenib dosage reduction[21].

In recent years, it has been pointed out that sarcopenia is a systemic disease. Cardiac sarcopenia likely manifests as a heart failure with preserved ejection fraction[1]. Relationships between heart failure and depleted lean muscle mass are indisputable and go both ways. Increased mortality in sarcopenic patients could be partly explained by this bilateral effect, especially in the posttransplant period. Cardiac ultrasound (US) is suggested to assess cardiac function in a sarcopenic patient with cirrhosis[22]. Involvement of the diaphragm leads to reduced peak cough flow in the elderly, increased rate of respiratory infections through impaired airway clearance and difficulties when weaning sarcopenic patients with cirrhosis from mechanical ventilation [5]. Impaired function of the cardiac and respiratory muscles likely contributes to clinical manifestations of dyspnea, weakness, fatigue, reduced exercise tolerance, and loss of appetite, all of which can further contribute to the propagation of sarcopenia and frailty[1].

DIAGNOSTIC METHODS IN THE FIELD OF SARCOPENIA

Questionnaires

Questionnaires take precedence over other tests because of their relatively short evaluation time and their possibility to monitor the condition in a dynamic timely manner[7]. Across the investigative options to assess malnutrition, Subjective Global Assessment (SGA) is the most frequently mentioned. Features of the SGA include a physical exam component that evaluates the loss of subcutaneous fat, peripheral or sacral edema, and muscle wasting. The quantity of muscle and subcutaneous tissue is graded subjectively by the examiner who then categorizes it as normal, mildly, moderately, or severely decreased. Multiple components on patient history are also evaluated. The first component is the amount of weight loss in the previous 6 mo. Supplementary historical features of the SGA include patient's dietary intake and the presence of gastrointestinal symptoms experienced daily for at least 2 wk. Once the history and physical examination sections are completed, patients are classified as well nourished (SGA grade A), moderately malnourished or suspected of being malnourished (SGA grade B), or severely malnourished (SGA grade C). SGA is a partially subjective method constituted by quantitative and qualitative variables, subject to varied interpretations, and reported as having low sensitivity in cirrhotic patients, as it can underestimate the nutritional state in their population[22].

Nutritional Risk Screening 2002 (NRS-2002), Liver Disease Undernutrition Screening Tool (LDUST), Royal Free Hospital-Nutritional Prioritizing Tool (RFH-NPT), and Malnutrition Universal Screening Tool (MUST) can be also used to assess malnutrition in hospitalized patients.

The NRS-2002 is a nutrition screening tool recommended by the ESPEN guidelines [17]. It includes three components—the nutritional score (BMI, weight loss, and dietary intake included), the disease severity score, and the age score (age > 70 years)[17,23]. Patients are classified as having no or low risk.

The LDUST assesses 6 factors that were identified as having the strongest associations with malnutrition in patients with chronic liver disease (nutrient intake, weight loss, loss of subcutaneous fat, loss of muscle mass, fluid accumulation, and a decline in functional status). The three potential patient responses are labeled and indicated as no signs of malnutrition, "mild to moderate" malnutrition, and "moderate to severe" malnutrition[24].

The RFH-NPT is a nutrition screening tool developed in the United Kingdom. It includes three major steps: (1) Patients who have alcoholic hepatitis or are undergoing tube feeding are immediately evaluated as high risk without proceeding to the next step; (2) Patients who do not have alcoholic hepatitis and are not undergoing tube feeding are assessed for fluid overload and its impact on food intake and weight loss; and (3) Patients who do not have fluid overload are assessed for nutritional status (BMI, unplanned weight loss, and daily dietary intake). Patients are stratified as being

at low, moderate, or high risk[2,25].

The MUST includes three categories: Current BMI, unintentional weight loss, and the presence of any acute disease that could compromise nutritional intake for more than 5 d[25].

A large study assessed the importance of RFH-NPT in the Asian population with predominantly viral liver cirrhosis. The questionnaire proved to be useful in the group of patients with a low MELD score and for assessing the prognosis of the disease. The disadvantage of the study is that different questionnaires are only compared with each other, and with only basic laboratory and anthropometric parameters[25]. In a recent publication, Buchard *et al*[5] noted that RFH-NPT and LDUST, despite recommendations for their use in patients with liver cirrhosis, have not yet been associated with clinical issues such as survival and complications appearance. Moreover, LDUST is based on the patient's statement and lacks objective data.

Laboratory tests

Myostatin is a natural muscle growth inhibitor. As a marker, it depends on gender and inflammatory processes in the body. In men with liver cirrhosis, it can be used to assess muscle mass, prognosis of decompensation events, and fit for surgery status. In contrast to Oshida *et al*[27] who came to different conclusions in the group of patients with compensated disease, the cited study used CT sarcopenia indices in a large group of patients with decompensated advanced chronic liver disease (dACLD). The limitation of the test is the decrease in the level of serum myostatin at a very low muscle mass stage which presents a major problem when defining possible cut-off values[26].

Another potential biomarker is irisin, a myokine, mainly expressed and secreted by skeletal muscles as well as functioning as an adipokine. A significant lower irisin level is proved to be a marker for muscle weakness and atrophy. In the group of patients with dACLD, the cited study demonstrated higher irisin levels in women without evidence of an association with irisin levels and the degree of hepatic impairment according to CHILD/MELD score, or the presence of ascites. Sarcopenia was assessed using a hand grip test and CT assessment of muscle mass[2]. In a related publication, a group led by Zhao *et al*[10] studied irisin levels in a group of patients with liver cirrhosis where a hand grip test and CT-assessed muscle mass were also used for the evaluation of sarcopenia. The difference between the studies was in the CT index used, as the latter used the proposed gold standard (see below). Lower irisin levels were demonstrated in the group of patients with a higher CHILD score. They also defined that it is not entirely clear whether this is the cause or the consequence of sarcopenia. Deficiency of the article are poorly defined criteria for the diagnosis of liver cirrhosis [10].

If muscles are damaged by diseases or vigorous exercise, titin is decomposed by proteolytic enzymes, and various titin fragments are detected in serum and urine. One of the isolated fragments is titin-N. The cited study defined its urinary excretion in a group of patients with metabolic-induced fatty liver disease. The study had a well-defined control group, but poorly defined comparative indices (CT indices? muscle and liver elastography? US parameters of skeletal muscle assessment?) in the article itself. According to their observations, titin-N was negatively associated with the amount of muscle mass (higher level in urine correlated with lower skeletal muscle mass) and positively associated with the occurrence of muscle myosteatosis (higher level in urine correlated with higher levels of US-assessed skeletal muscle fat). They also identified an association of the biomarker with the degree of muscle fibrosis progression which may be associated with functional muscle decline. For the latter, a knee extension test and a hand grip test were used as a comparative test[27].

Anthropometry

According to EASL guidelines, two simple criteria can be used in everyday clinical practice to stratify patients at high risk of malnutrition: being underweight (BMI < 18.5 kg/m^2) and having advanced decompensated cirrhosis (Child C patients)[28]. In patients with ascites and peripheral edema, dry weight assessment is proposed, either estimated by the post paracentesis body weight, the weight recorded before fluid retention if available, or by subtracting a percentage of weight based upon the severity of ascites (mild 5%; moderate 10%; severe 15%), with an additional 5% subtracted if bilateral peripheral edema is present[25,28].

Of the measurements that can be performed with an ordinary tape measure, the most common are mid-arm muscle circumference (MAMC), mid-arm muscle area, and triceps skinfold (TSF), all of which are simple and rapid to perform low-cost tests that are not affected by the presence of fluid retention[7,28]. Some Asian studies mention

calf circumference which showed a good association to the frailty[29] in their study population and for the assessment of sarcopenia in the group of patients with compensated ACLD (cACLD), or in patients without ascites[30]. The disadvantage of both studies is the assessment of muscle mass with tests that are less useful in the overweight or hypervolemic patients' population.

Simple tests of muscle function

This group of tests can be performed at the bedside or during outpatient consultations.

The Short Physical Performance Battery consisting of three methods - balance test, gait speed test, and five chair stand test - can be used to assess the patient's functional ability[2,7].

Handgrip strength test (HS) is the most commonly studied test in this group using a calibrated dynamometer. The test depends on the patient's age and BMI. An interesting study has shown that HS in combination with the MELD score in men awaiting liver transplantation can be superior to CT modality if mortality was observed as a clinical outcome. Study results could be explained due to the early decline in muscle function even before the decline in muscle composition and mass occurs[31].

To assess muscle function, a test cited by Buchard *et al*[5] is commonly mentioned - the Liver Frailty Index (LFI). Its role is to evaluate frailty by combining HS, chair stands, and balance tests. Using a provided cut-off, LFI was associated with mortality independently of the presence of HE and ascites[5,7].

The next frequently used test is a six-minute walk test which has a sensitivity of 90% for identifying patients with increased risk for pre-LT mortality when performed at less than 250 m[1,7].

A walking speed/gait test is offered as a third option[2,32]. In a study by Nishikawa *et al*[32], a 6 m walking test was performed to measure muscle function in a group of patients with liver cirrhosis, with walking speed (WS) and gait speed (GS) defined, respectively. As a reference to define sarcopenia, bioelectrical impedance analysis (BIA) was used and therefore only patients without ascites were included. They observed that improvement in WS requires quick movement, whereas the improvement in the GS does not. WS requires maintained cognitive function, and muscle strength may be necessary for its improvement, while the GS value will not decrease as long as muscle strength is maintained even if cognitive function is reduced. They confirmed that hypervolemia or tissue edema and the presence of various orthopedic diseases have a significant effect on both tests. It is likely that WS could have a greater impact on the assessment of frailty than on the assessment of sarcopenia[32].

Body composition measurements

These diagnostic methods represent the foundation of modern clinical body composition analysis but with several important limitations when evaluating patients with liver cirrhosis.

BIA: A fixed, low-voltage, high-frequency alternating current is introduced into the human body to assess body electrical conductivity together with resistance (impedance). Subsequently, capacitance is the parameter that makes the current lag behind the voltage, which results in a phase shift. This shift is measured geometrically as the angular transformation of the capacitance to resistance ratio, or the phase angle (PA). BIA measures belong to safe, rapid, easy-to-perform, and quite accurate methods of estimating fat mass and fat-free mass. The main limitation of its use in the group of patients with liver cirrhosis is the influence of hypervolemia, physical activity, use of diuretic therapy, BMI, and liquid or food intake before the test[13,24,33].

There are several parameters that can be measured by means of BIA: Body cell mass, total body water, extracellular water, extracellular mass, and body fat. Multifrequency BIA analysis has been proposed lately because it is less influenced by overhydration. It measures the above-mentioned parameters by passing a series of different electrical currents and electrical frequencies through the body. Segmental BIA can also be used to overcome the fluid retention bias[33].

PA was observed to be less affected by overhydration while being a reliable indicator of clinical outcome. As such, a PA result less or equal to 5.4 degrees was a predictor of reduced survival in one of the conducted surveys. The data obtained from another investigation indicated PA cut-off less than or equal to 4.9 as a predictor of disease progression and mortality in cirrhotic patients[17,33]. A Japanese study comparing the applicability of BIA to equivalent anthropometries (MAMC, TSF) found that BIA measures comparably 6%-16% higher values of muscle mass estimates. Nevertheless, the results coincided with the prognosis of mortality in the cohort where

cirrhosis was proven histologically in all patients. The disadvantage of the study is the inclusion of mostly compensated patients, and that no comparison with other tests was performed[18].

Dual energy x-ray absorptiometry: Dual energy x-ray absorptiometry (DEXA) uses low-dose x-rays to provide a comprehensive 3-dimensional analysis of the entire body, thus automatically breaking down each body compartment into bone mass, fat mass, and fat-free (or lean) mass. It is safe, inexpensive, readily available, and reproducible. In comparison to the CT scan, it uses less radiation. As with BIA, the problem of analysis may be water accumulation in muscle and fibrous tissue, especially in the elderly. Also, it is difficult to access in some medical centers[13,31,33].

In the group of patients with liver cirrhosis, it shows an association between muscle mass assessment and mortality[35]. Appendicular lean mass (APLM) has been proposed as the most appropriate method in cirrhosis to minimize confounding by ascites [31]. A well-designed Greek study that excluded the population of patients with active alcohol consumption compared the proposed APLM index (ALMI - APMI balanced to the patient's height; unit kg/m^2) to CT indices of muscle mass, together with multivariate analyses of patient's age and gender. They compared the success of detecting sarcopenia by DEXA and by the CT cut-off values proposed by several study groups (Carey, Montano-Loza, Mourtzakis, ESPEN, and indices proposed from oncological populations - Martin, Prado). They found that the proposed cut-off values in the DEXA-assisted analysis matched the detection of sarcopenia very well when comparing the CT cut-off values proposed by the various research groups[36]. An important finding of the study was also that the prevalence of low muscle mass did not reveal any statistically significant difference in relation to disease etiology in general. However, it should be noted that a significantly higher percentage of patients with an alcoholic etiology was found in the low muscle mass subgroup compared to the normal muscle mass group. As a weak point of the DEXA analysis, they mention the accessibility of the investigation in smaller hospital centers[36].

On the other hand, some studies report only a weak concordance between DEXA and CT when identifying sarcopenia in cirrhosis[24]. The rest state that DEXA indices show a sex-related distribution of body compartments. In cirrhotic women, more reduction in fat stores is observed with the maintenance of lean tissue. In men, the loss of lean tissue is the most featured early phenomenon. A described pattern is reflected by a weak association between muscle strength and muscle mass in cirrhotic women [33].

The following study in this area states the possibility of using proposed DEXA limb muscle mass indices which showed a good correlation compared to CT indices. The study was conducted in a small cohort of patients awaiting liver transplantation. They observed that fewer women were identified sarcopenic with DEXA than expected. They also cite the well-known DEXA deficiency as not being able to offer muscle structure quality determination compared to the CT examination[37].

US: US can be used to evaluate echogenicity, diameter, cross-sectional area, and muscle volume. Performing three consecutive measurements and using their mean value as a final result is proposed. The patient should rest for at least 5 min before the measurement and not exercise less than 30 min before the examination. To assess the muscle contraction potential, muscle thickness can be measured before and after the movement, especially in muscles that have a significant change in diameter during contraction. The possibility of assessing microvascularization with contrast enhanced US and the assessment of pennation angle are mentioned. A special expert group for this field lists 39 muscle groups that can be evaluated using US examination. They propose standardizations of the measurement site and explore main problems of the investigative methodology that should be solved. The inability of assessing some muscle components with US is the most common problem. Other problems mentioned are visibility, dependency of the operator's experience as well as the patient's general condition impact on the measurement value, and its dependency on the equipment quality[38].

In this area, the quadriceps muscle evaluation, especially its thickness and quality, is most often cited in the literature. Pita *et al*[39] estimated a daily decline in muscle mass in patients with cirrhosis who were waiting for a liver transplant in the intensive care unit. In a relatively small cohort, CT-estimated muscle mass was used for comparison demonstrating the ability of the US to monitor muscle mass decline with daily measurements of rectus femoris muscle diameter, as well as the association between this result and mortality in the studied patients. Another frequently cited study from this area performed quadriceps diameter measurement and proposed a model that included BMI. It states that the US of quadriceps muscle is a low-cost, reliable,

reproducible, and accurate estimate of muscle mass that can be completed at the bedside or in an outpatient clinical setting as well as repeated without concern of radiation exposure. Gender-specific nomograms that correlated well with CT control were suggested. The cohort of patients was small, but the comparability between the two operators was good[40].

Measurements of the tongue muscle thickness in the group of patients with liver cirrhosis showed an association with the CHILD and MELD score, and a distinctive difference between the group of healthy control group, but with no proven CT control correlation. The definition of liver cirrhosis in the article was relatively loose[41].

In a population of Japanese cirrhosis patients, Kobayashi *et al*[42] measured the area of the psoas muscle in the right groin area and balanced it by the square of body height. The examination was quick and performed at bedside in a large cohort of patients. The study showed a significant correlation between US and CT measurements, suggesting the reliability of the US measurement. In addition, the proposed US index was significantly associated with CT obtained measurements. There was also a good comparison between the two investigators and 100% applicability of the method in the investigated cohort. A small number of patients with dACLD, especially patients with ascites, can be mentioned as a study deficit[42]. Our group also used the psoas muscle measurement in a small group of patients with dACLD. The inferolateral area in respect to the right kidney was used as the measurement site and the estimated psoas muscle diameter was balanced to the patient's height. The proposed index showed clinical significance when predicting any subsequent decompensation and patient mortality. The measurement showed an important limitation in terms of practical use, as it was not feasible in about a quarter of patients due to technical limitations (ascites, obesity, visibility). Also, no comparison with any of the standard investigatory methods was made[43].

In recent years, the use of elastography has been suggested to assess muscle stiffness which could indirectly point to the loss of muscle function. A well-structured study in a small cohort of patients showed an association between femoral muscle elastography results and frailty[44].

The US imaging of some of the above mentioned methods is showed in Figure 2.

Cross-sectional imaging: CT: CT allows an accurate cross section assessment of muscle mass area and an estimate of tissue density using Hounsfield units (HU)[13]. It is recommended to use tissue that shows HU estimated density from -29 to +150 on two consecutive CT slices at L3 level. To make it more clinically applicable, it is suggested to use various computer programs which automatically and very reliably evaluate the muscle area (*e.g.*, SliceOmatic, ImageJ, FatSeg, OsiriX)[7]. All these programs require manual image analysis by a radiologist. In this regard, some studies have tested the concept of a methodology to measure psoas muscle features on incidental CT scans using an automated, deep convolutional neural network model, a technology routinely used for facial recognition software. Automated measurement in the cirrhosis patient population proved to be comparable to that where the measurement was performed with standardized programs. It was significantly faster and independent of human error[45]. An alternative option is open-source software for image processing which requires manual tracing of various abdominal regions to obtain body composition information. Using these results and a preprogrammed template, the operator can easily generate various muscle indices. Alternatively, the use of the method first published by Durand *et al*[35] (see below) is suggested[45].

The most common shortcomings of CT sarcopenia analysis are unclear possibility of recurrent/dynamic evaluation due to radiation exposure and the impact of the result on clinical decisions; unclear protocol of the examination method; radiation impact; examination costs; and the need for additional software to analyze images. In addition to the ability to differentiate three main body compartments, *i.e.*, muscle, visceral, and subcutaneous adipose tissue, the ability to identify muscle radiodensity to determine ectopic fat accumulation in muscles, and the fact that it is probably not affected by the presence of ascites or edema, the pros are the relative ease, speed, accuracy, and accessibility of the examination in a hospital setting. There is a strong possibility of price and radiation exposure reduction to only 2.6 mSv by a single slice CT, and CT shows a very good reproducibility between different performers[7,19,31].

The test of choice is the L3 skeletal muscle index (SMI), a muscle area on a CT scan at the level of the L3 vertebrae corrected for height (Figure 3)[7]. Carey *et al*[24] recently defined sarcopenia as SMI < 50 cm^2/m^2 in males and 39 cm^2/m^2 in females in a cohort of cirrhotic patients in the North American region awaiting liver transplantation[1,13,24]. In this cohort, a CT measured SMI was a strong surrogate of whole-body muscle mass and can be applied as a reliable marker of the whole-body mus-

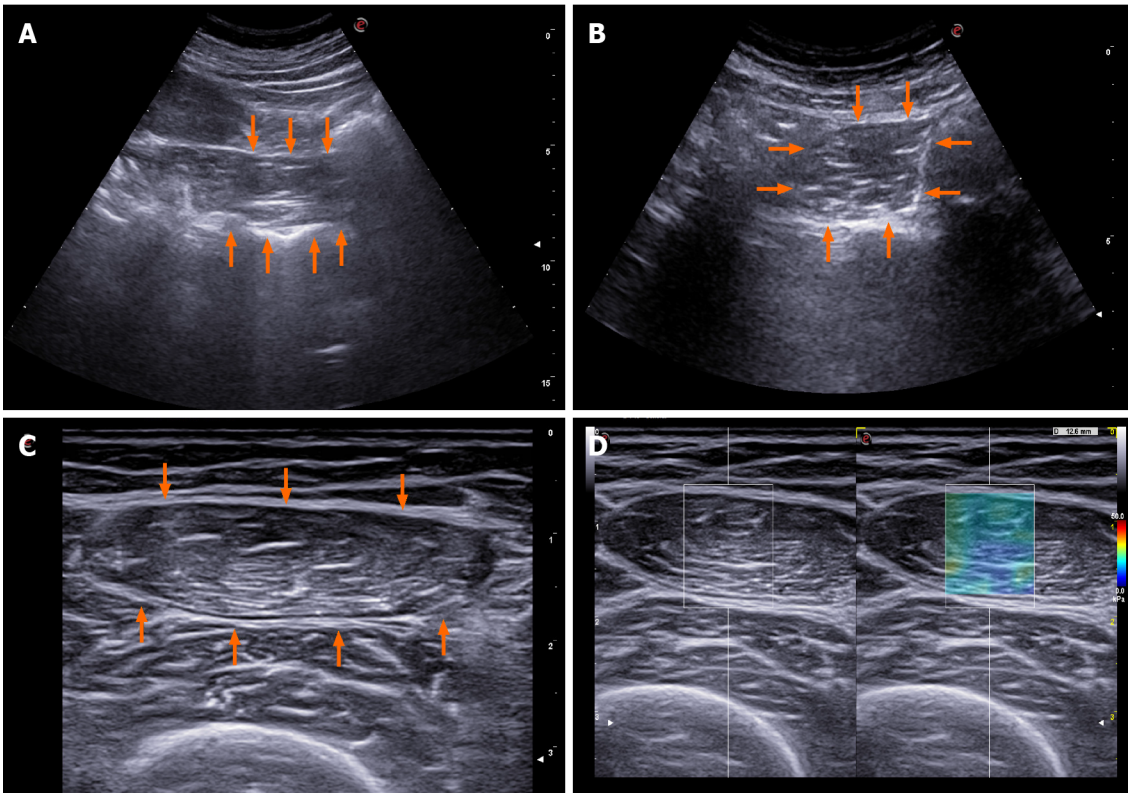


Figure 2 Reported options for ultrasound muscle mass assessment in cirrhosis. A: Psoas muscle evaluation as proposed by Hari *et al*[43]; B: Psoas muscle evaluation as proposed by Kobayashi *et al*[42]; C: Femoral muscle evaluation as proposed by Tandon *et al*[40]; D: 2D-shearwave elastography of the femoral muscle.

cularity. Defined in this way, sarcopenia was mainly delineated as sex-specific SMI values associated with mortality that were independent of age and MELD score[13,24]. When assessing sarcopenia by SMI on an abdominal CT scan, there does not appear to be a large difference between measurements at L3 *vs* L4 vertebrae[10]. There is also an excellent agreement between various software programs with respect to measurements of abdominal skeletal muscle area[46].

SMI seems to be a more complete and robust measurement than individual measurement of the psoas muscle or the psoas muscle index (PMI), especially in men with cirrhosis. In addition, low PMI identifies an incomplete subset of patients at increased risk of mortality indicated by low SMI[13,47,48]. The degree of sarcopenia can also be defined by SMI as severe sarcopenia [SMI two SD below the sex-specific mean value of a young donor (18-40 years old), sarcopenia (SMI between -1 and -2 SD of sex-specific young) donor mean values] and as a non-sarcopenic group (SMI higher than one SD below the sex-specific mean for young donors)[49]. SMI study validations include numerous publications. Important study of this area published by Ebadi *et al*[49] defined cut-off values compared to healthy controls. In their cohort, cut-off values for SMI were < 42 cm^2/m^2 in men and < 30 cm^2/m^2 in women (for severe sarcopenia) and < 50 cm^2/m^2 and < 37 cm^2/m^2 for sarcopenia. Mortality was considered as a clinical outcome. The cohort showed the presence of severe sarcopenia in 8% of patients, the majority of which had alcohol-related cirrhosis[49].

The second common cited choice is the index first proposed by Durand *et al*[35]. They demonstrated that the ratio between the transverse diameter of the psoas muscle measured at the L3 level (umbilicus is suggested as a reference point) and balanced for the patient's height [psoas to height ratio (PTHR); unit mm/m] is an objective indicator of muscle loss and a predictor of mortality in patients with cirrhosis, independent of MELD or Na-MELD score. In their retrospective study, they also demonstrated the effect of the PTHR on mortality in patients with refractory ascites [35]. A study by Paternostro *et al*[47] tested the value of a similar index. They demonstrated the clinical applicability and calculative ease of PTHR to define sarcopenia with similar cut-off values as proposed by Durand *et al*[35]. A weak point of this methodology is a variable landmark (the umbilicus) to measure muscle thickness; thus, CT-derived index from single muscle measurement can still not be recommended[46].

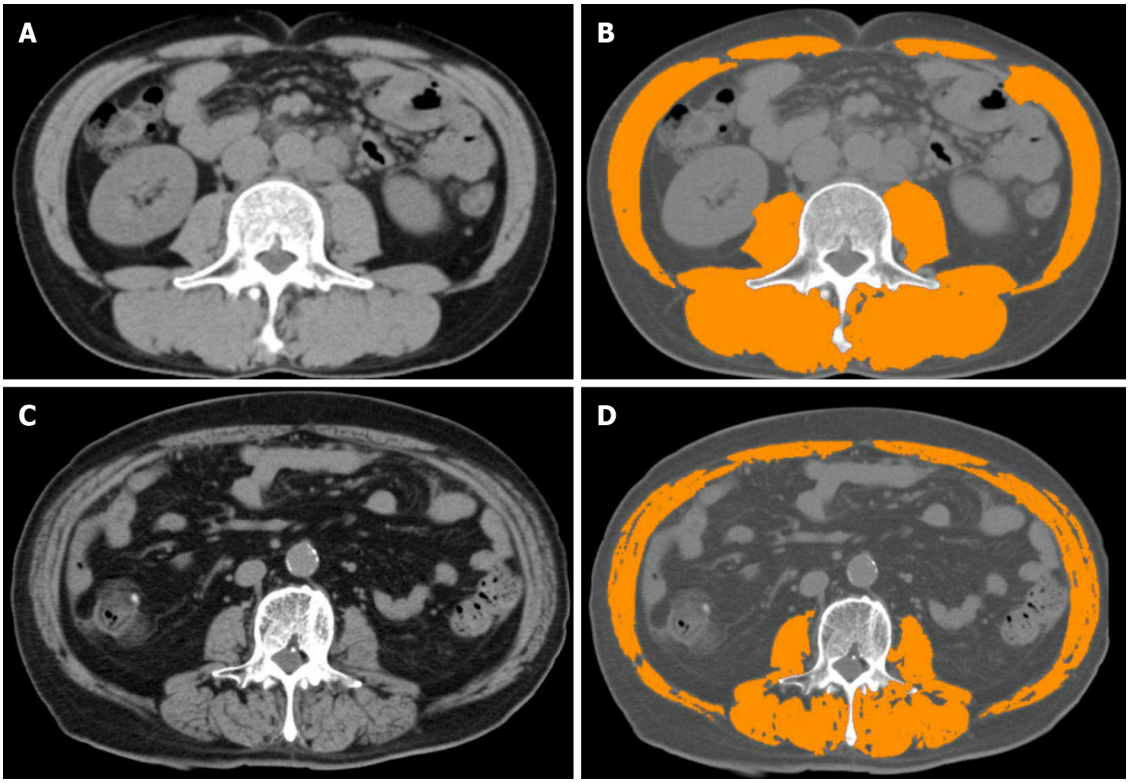


Figure 3 Computed tomography modality and skeletal muscle index. A and C: Analysing computed tomography slices obtained at the third lumbar vertebra level; B: Patient with normal skeletal muscle index (SMI) values; D: Patient with reduced SMI values. After using medical imaging software and analysing areas of predefined Hounsfield units, SMI values are calculated.

To determine myosteatosis, CT should be performed without the use of a contrast agent. Muscle density is assessed by HU values[13]. To define myosteatosis, the proposed cut-off values are > 41 HU in patients with BMI < 24.9, and > 33 HU in those with a BMI > 25. In a group of patients with liver cirrhosis, Bhanji *et al*[50] demonstrated the effect of CT-assessed myosteatosis on the occurrence of HE within one year of the measurement. It is probably a risk independent of sarcopenia.

An interesting option offered by CT is the already mentioned possibility to assess the concomitant presence of sarcopenia and myosteatosis. In a small cohort of patients with cirrhosis, Nardelli *et al*[51] assessed the presence of sarcopenia and myosteatosis with suggested CT SMI and HU values. Minimal HE defined by psychomotor tests was more common in the group of patients with both factors, as was elevated blood ammonia.

Magnetic resonance imaging: Magnetic resonance imaging (MRI) is considered as an appealing test for the diagnosis of muscle wasting due to the lack of radiation exposure and high-quality images, including information on muscle quality as evident by fat infiltration[7]. When comparing results from CT and MRI images in Traub *et al*[8] study cohort, there was no difference in the detection rate of reduced muscle mass. However, the study did not compare CT and MRI images in the same patients since they used only imaging studies that were routinely performed. The study by Beer *et al*[52] included patients with cirrhosis who had clinical or imaging cirrhosis parameters. Additionally, the FIB-4 score was used to assess the degree of liver fibrosis. Portal hypertension was defined invasively, or its signs were evaluated by gastroscopy. MRI-assessed sarcopenia using PTHR index demonstrated an association between sarcopenia rates and mortality in patients with cACLD and the influence of sarcopenia on infection or mortality due to infections. Mortality was particularly high in the PTHR < 8 mm/m (female) and < 12 mm/m (male) group. They also pointed out the advantage of the MRI for its possibility to provide sarcopenia data without the use of a contrast agent and without radiation exposure and reported good comparability with CT-PTHR measurements as well as good agreement between the two readers[52].

MRI offers another imaging technique, namely diffusion weighted imaging (DWI). DWI offers additional information regarding the composition and architecture of investigated tissue. DWI quantified by apparent diffusion coefficient (ADC) can reflect different pathological changes, such as cell density, extracellular matrix, nucleic areas,

and membrane permeability, and may have a role in the diagnosis of different muscle disorders. In the cited study, ADC maps were thus created by the implemented software and manually drawn regions of interest on the ADC maps along the contours of the iliopsoas and paravertebral muscles to avoid fat areas and vessels. They confirmed that ADC can reflect pathological muscle changes since myositis and myopathy had statistically significantly higher ADC values in comparison to unaffected muscles. The study does not offer a direct proposal for the clinical use of the proposed indices in the assessment of sarcopenia. In the multivariate analysis, the authors also used only the MELD score[53].

CLINICAL TREATMENT OPTIONS

The treatment of sarcopenia is focused on drug treatment possibilities, exercise and dietary measures, and the treatment of decompensated liver cirrhosis. There are currently no studies that prove the benefit of one or the other in terms of reducing mortality. There is a general benefit of the interventions in terms of reducing the feeling of fatigue; increasing vital capacity; improving muscle mass and ability to exercise; and last but not least, improving quality of life[7].

The general objectives of drug treatment are to focus on lowering blood ammonia and improving the action of growth hormone and testosterone in certain parts of the population with liver cirrhosis. Special emphasis should be placed on the elimination of poor appetite and complete absence of physical activity[1,2,7]. Studies suggest a possible beneficial effect of testosterone on improving muscle mass and function without significant side effects of treatment, but the evidence is yet not enough to recommend replacement therapy[2,7]. Extensive study data on the use of growth hormone in this area are not available. The importance of regulating thyroid hormone and blood sugar levels is also mentioned[24].

General nutrition advice concerns sufficient energy and adequate protein intake while avoiding prolonged fasting periods (> 6 h). The patient should eat three to five meals a day while the target caloric intake varies according to the patient's BMI. As per EASL guidelines, at least 35 kcal/kg of actual body weight per day is recommended in non-obese individuals (BMI < 30). Second, all patients should be provided with a target protein intake - guidelines have been consistent in the recommendation of 1.2 g/kg to 1.5 g/kg per day. This can be achieved by using multiple sources including meat, dairy, and vegetable proteins, with some support from the literature stating that the latter two sources may have additional benefits against HE. In obese patients with cirrhosis, a moderate decrease in caloric intake (500 kcal to 800 kcal daily and not less than 70% of previous caloric intake) with a tailored, individualized dietary plan is recommended. Best results on liver histology and on the HVPG were observed in patients achieving a weight loss of 10%, and this should probably be considered the target of lifestyle interventions. A late evening snack before night sleep with 50 g of complex carbohydrates and approximately 15 g of protein content is acceptable and significantly increases muscle mass and may also have beneficial effects on HE reccurence. Patients should be counseled on the need to eat breakfast[11,13]. Evidence is mounting regarding the benefit of Mediterranean diet -rich in vegetables, fruit, and olive oil - in the cirrhotic population. In this fashion, patients are advised to avoid processed foods and use fresh ingredients[11,17]. Among the dietary supplements, the daily use of branched chain amino acids and aromatic amino acids which are probably associated with event-free life and a quality of life is much mentioned. Special attention is also paid to the effect of L-carnitine, vitamin D, omega-3 fatty acids, and leucine[1,7,13]. Vitamin and microelement deficiency should be excluded, and in case of a deficit, vitamin D and zinc should be supplemented. Branched chain amino acid supplements and leucine-enriched amino acid supplements (6-8 g per day) should be considered, particularly in decompensated patients[11]. Ghrelin, a peptide produced in the stomach, has anabolic and anti-inflammatory properties that make it a promising agent[10]. A small study assessing ghrelin infusion in patients with heart failure showed improvement in LV function, muscle strength, lean body mass, and exercise capacity[1].

In the field of exercise, moderate intensity exercise is recommended for at least 30 min per day, 3-5 times per week. Physical exercise should start with a short-term warm-up and end with a stretching/cool-down phase. Although a combination of resistance and aerobic exercise is recommended, resistance exercise is more effective in reversing sarcopenia[9,13]. Avoidance of sedentary behavior should be recommended even in patients not willing to undergo a formal exercising program to increase daily

physical activity within the context of NEAT (nonexercise activity thermogenesis). As opposed to scheduled exercise, NEAT encourages patients to take opportunities to increase their activity within their day-to-day activities[11]. Exercise brings improvement in insulin sensitivity, an increase in muscle protein synthesis, and the use of muscle as an alternative route for ammonia detoxification. According to EASL guidelines, the target for moderate aerobic activity is to eventually reach up to 150 min per week, incorporating resistance activities on two or more days per week, except for abdominal workouts which are not recommended as they might abruptly increase abdominal pressure[11].

In the general complications of liver cirrhosis treatment area, there is evidence of the beneficial effect of TIPSS insertion on sarcopenia[1,7]. Thus, an uncontrolled study found that the insertion led to an increase in muscle mass and an improvement in overall prognosis. It also showed an improvement in psoas muscle area in 70% of patients with an increase in the mean muscle area after TIPSS insertion[2]. As previously stated, an already present sarcopenia could be considered a risk factor for mortality in patients who undergo TIPS placement[3], data, confirmed with another retrospective study in patients with cirrhosis who undergo TIPS placement for refractory ascites[54]. The therapeutic consequences of this findings are yet to be provided. The final treatment option for many patients with advanced liver cirrhosis is, of course, liver transplantation, which has been shown to have a beneficial effect on reversibility of muscle function after liver transplantation[9].

Dilemmas remain regarding the timeline of the proposed measures. It is hypothesized that an earlier intervention at a time when anabolic potential exists may be more effective than an intervention at a refractory stage of muscle wasting. Studies in this area are rare and have mostly been performed in very small populations in the field of cirrhosis[3]. To date, there is no evidence that exercise or dietary supplements affect sarcopenia through dysbiosis[4]. There is also no clear guidance on the group of patients with HCC and liver cirrhosis where interventions regarding exercise and dietary substitutions before and after treatment would probably be beneficial[21].

CONCLUSION

From their repeatability, comparability, accessibility, and realistic range for the correct clinical assessment of an individual patient, the main qualitative problem of sarcopenia assessment in the population of patients with liver cirrhosis is the great heterogeneity of the proposed clinical tests (Figure 4). Control tests in clinical trials that are not generally proposed to assess sarcopenia in patients with liver cirrhosis are also common. From the patient's point of view, correct assessment of the sarcopenia degree in the female population as well as in the population of overweight patients further exists. The unanswered question is the possibility of transferring study-confirmed findings between different races. When analysing different published studies, it is common that they address study issues within a small cohort that is occasionally incoherent or too isolative. Many studies are retrospective and represent an analysis of a difficult to verify or an incomplete mass of data stored in hospital computer systems rather than in study-specific storage programs. A common problem is the definition of the liver cirrhosis diagnosis which should in modern cACLD treatment be mostly elastographic, but certainly not a result of the ICD coding nomenclature. All with the aim to cover enough patients in the early disease stages and to discern patients who have sarcopenia at the expense of actual chronically impaired liver function. On the other side of the spectrum, many studies with sarcopenia do not pay enough attention to the population of patients with permanent decompensated disease, especially to those with refractory ascites. Many authors also point out that sarcopenia studies rarely include tests to define muscle function and focus solely on static information regarding total muscle mass which is easier to perform from the study complexity point of view. In this area, the question remains unanswered as to what is the long-term relationship between muscle mass and function, which is probably not linear throughout, and whether this relationship has a significant impact on the risks associated with the presence of sarcopenia. There is considerable incoherence in the studies regarding the use and comparison of different (especially CT-defined) indices of muscle mass or function as well as the inclusion of own study-defined claims in sequential studies (possibility of continuing primary study error).

The definition of CT-SMI as the gold standard for muscle mass assessment and the efforts of several study groups to define valid cirrhosis related sarcopenia thresholds are an important foundation for the future study and clinical applicability of this field.

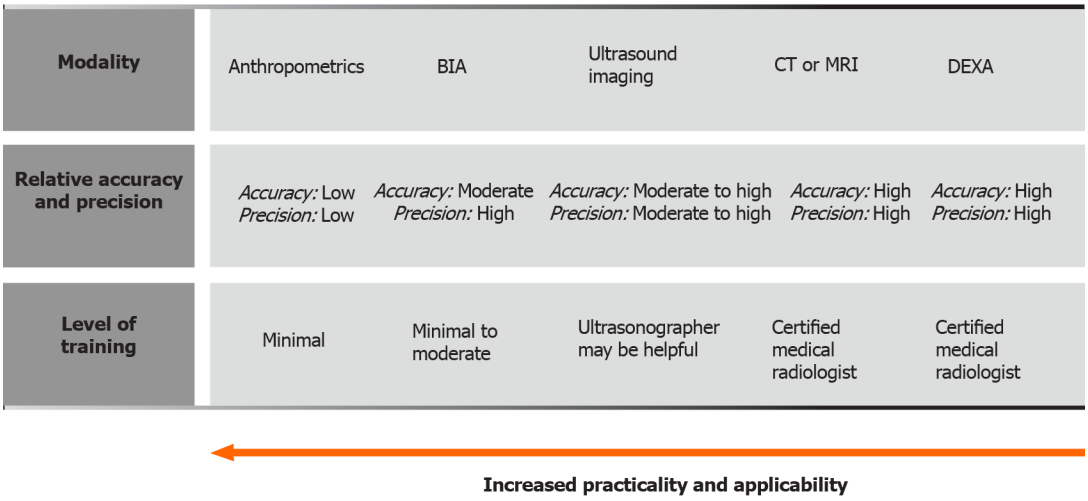


Figure 4 Sarcopenia evaluating methods. Distinctions regarding required training, applicability, practicality, accuracy and precision are presented. Adapted from Tandon *et al*[55]. BIA: Bioelectrical impedance analysis; CT: Computed tomography; MRI: Magnetic resonance imaging; DEXA: Dual energy x-ray absorptiometry.

Indeed, some data suggest that even small changes in the cut-off values could mean very relevant shifts in the detection and study evaluation of sarcopenia in this population[8].

Study-malnourished areas in the field of sarcopenia and liver cirrhosis are the area of sarcopenia-CSPH interdependence, the area of vulnerable groups (HCC patients, alcoholism, morbid obesity), and the influence of sarcopenia on clinical outcomes in addition to mortality, especially on various forms of disease decompensation. A small number of studies pays attention to the field of sarcopenia treatment through the therapeutic interventions. Such studies are, of course, necessary for the long-term clinical applicability of the field but difficult to perform in larger cohorts.

According to certain clues, sarcopenia could have an important decision regarding the clinical choice for liver transplantation, both in terms of the patient's higher priority and in terms of the patient's ability to be fit for a major surgery. In countries that allow such graft allocation, this could lead to the selection of poorer transplants or living donor transplants, or to the rejection of the transplant process in the event of an estimated poor yield.

At the very end, an issue remains that we as clinical professionals find difficult to face. Does the current definition of sarcopenia by the above-mentioned tests have any relevant clinical implications regarding patients' survival? Although most of the answers point to the affirmative, we are still quite a long way from the objective long-term goals of successful treatment in this area.

Nevertheless, we can certainly be justifiably pleased and proud to look at the huge leap of the last decade in terms of the knowledge gained in this clinically most relevant field. New possibilities for the use of existing and more modern indices of declining muscle mass and function in a patient with cirrhosis are coming to the fore. The tests mentioned in the article are increasingly striving for repeatability and simplicity, both in enabling dynamic sarcopenia monitoring and in reducing investigator dependent errors. A realistic desire for a study breakthrough in the upcoming years remains to define sarcopenia tests and indices that would ensure further ease of affordability and mass clinical applicability for a patient with liver cirrhosis.

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ORIGINAL ARTICLE

Basic Study

Impact of *Fusobacterium nucleatum* in the gastrointestinal tract on natural killer cells

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Abstract

BACKGROUND

Gut microbial dysbiosis contributes to the development and progression of colorectal cancer (CRC). Natural killer (NK) cells are involved in early defense mechanisms to kill infective pathogens and tumor cells by releasing chemokines and cytokines. To better understand the relationship between the gut microbiome and CRC, it was hypothesized here that a high abundance of *Fusobacterium nucleatum* (*F. nucleatum*) in the gastrointestinal tract could cause reduced NK cell activity.

AIM

To identify associations between gastrointestinal tract *F. nucleatum* levels and NK cell activity.

METHODS

In vitro experiments were performed on NK cells treated with *F. nucleatum, Peptostreptococcus anaerobius,* and *Parvimonas micra* to identify the effects of gut microbiome species on NK cells. Following 24 and 48 h of treatment, NK cell counts were measured. In parallel studies, C57BL/6 mice were given broad-spectrum antibiotics in their drinking water to reduce resident gut flora. After 3 wk, the mice received the various bacterial species or phosphate-buffered saline (PBS) *via* oral gavage every 2 d for 6 wk. At the study end, blood samples were acquired to perform NK cell activity assessment and cytokine analysis. Intestinal tissues were collected and analyzed *via* immunohistochemistry (IHC).

RESULTS

The data show that after 3 wk of broad-spectrum antibiotic treatment, levels of total bacteria and *F. nucleatum* were markedly decreased in mice. Gavage of *F.*

manuscript.

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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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nucleatum significantly decreased NK cell activity relative to the activities of cells from mice treated with antibiotics only and PBS. The administration of *F. nucleatum* decreased the proportion of NK46$^+$ cells based on IHC staining and increased the production of interleukin-1β and tumor necrosis factor-α.

CONCLUSION

High levels of *F. nucleatum* in the gastrointestinal tract reduced NK cell activity in mice, and the decrease in NK cell activity might be affected by increased pro-inflammatory cytokines after *F. nucleatum* treatment.

Key Words: *Fusobacterium nucleatum*; Natural killer cells; Colorectal cancer

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Core Tip: Gut microbial dysbiosis contributes to the development and progression of colorectal cancer (CRC). Natural killer (NK) cells are involved in early defense mechanisms to kill infective pathogens and tumor cells by releasing chemokines and cytokines. To better understand the relationship between the gut microbiome and CRC, it was hypothesized here that a high abundance of *Fusobacterium nucleatum* (*F. nucleatum*) in the gastrointestinal tract could cause reduced NK cell activity. Accordingly, this association would impact the development of CRC in a host. To test this hypothesis, associations between the abundance of *F. nucleatum* in the murine gastrointestinal tract and NK cell activity were evaluated. In this study, we showed that a high abundance of *F. nucleatum* in the gastrointestinal tract reduced the activity of NK cells and that such outcomes might be associated with a concurrent increase in circulating levels of some pro-inflammatory cytokines, including interleukin-1β and tumor necrosis factor-α in these hosts. These findings suggest that increases in *F. nucleatum* in the gastrointestinal tract could be a factor in disrupting how the immune system prevents the development of CRC.

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INTRODUCTION

Increasing evidence indicates that gut microbial dysbiosis contributes to the development and progression of colorectal cancer (CRC)[1-3]. Studies have suggested that the presence of several bacteria, including *Fusobacterium nucleatum* (*F. nucleatum*), *Peptostreptococcus anaerobius* (*P. anaerobius*), *Parvimonas micra* (*P. micra*), and *Solobacterium moorei* are significantly associated with CRC[4,5]. Other studies have described a strong correlation between the presence of *F. nucleatum* (a common oral anaerobic Gram-negative rod that is a typical cause of periodontal disease) with CRC development and pathogenicity[6-8]. The presence of *F. nucleatum* increases the proliferation of CRC cells and tumor development in mice by activating Toll-like receptor 4 signaling to nuclear factor-kappa B, and up-regulating the expression of miRNA-21. Other studies have shown that *F. nucleatum* can suppresses the tumor-immune microenvironment in a manner that could inhibit T-cell responses to antigens and arrest human T-cells in the G_1 phase or induce apoptotic cell death[9-11]. In one report, the Fap2 protein of *F. nucleatum* was shown to play a crucial role in mediating fusobacterial CRC enrichment by Gal-GalNAc that is over-expressed in CRC[6]. In addition, this protein directly interacts with the inhibitory receptor TIGIT on natural killer (NK) cells, leading to an inhibition of NK cell cytotoxicity in humans[8].

NK cells are large granular lymphocytes (unique type of cytotoxic lymphocyte) that play a role in innate immunity[12]. NK cells are involved in early defense mechanisms to kill infective pathogens and tumor cells by releasing chemokines and cytokines, as well as by helping other immune cells in targeted cell elimination[13]. Imai *et al*[14] in a follow-up study of 154 patients with cancer for 11 years found that patients with

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high cytotoxic activity among their peripheral blood lymphocytes had decreased cancer risk and, conversely, those with low cytotoxic activity had increased cancer risk [14]. In a study that evaluated 140 patients with Stage III CRC who underwent surgery, it was seen that lower pre-operative NK cell activity resulted in significant effects on the development of metachronous distant metastases[15]. A cross-sectional study performed in Canada that evaluated NK cell activity in a high-risk population of subjects screened for CRC (*via* colonoscopy) showed that subjects with CRC had lower NK cell activity compared to those without CRC[16]. In addition, NK cells have been shown to be deficient when analyzing their localization and densities within CRC tissues[17].

To better understand any relationship between the gut microbiome and CRC, it was hypothesized here that a high abundance of *F. nucleatum* in the gastrointestinal tract could cause reduced NK cell activity. Accordingly, this association would impact upon the development of CRC in a host. To test this hypothesis, associations between the abundance of *F. nucleatum* in the murine gastrointestinal tract and NK cell activity were evaluated.

MATERIALS AND METHODS

Bacterial culture

F. nucleatum (*ATCC 25586*), *P. anaerobius* (*ATCC 27337*), and *P. micra* (*ATCC 33270*) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All bacteria were cultured at 37°C in Anaerobe basal broth (CM0957, OXOID, Thermo Fisher Scientific, West Palm Beach, FL, USA) in an anaerobic jar (Hardy Diagnostics, Santa Maria, CA, USA). Anaerobic conditions were maintained using AnaeroGen packets (Thermo Fisher Scientific).

Culture of NK cells

For *in vitro* studies, NK92 cells (CRL-2407, human NK line) were obtained from ATCC. The cells were cultured in a minimum essential medium (MEM, Sigma, St. Louis, MO, USA) containing 12.5% fetal bovine serum (HyClone, Logan, UT, USA), 12.5% horse serum (HyClone), 200 U recombinant IL-2/mL (Invitrogen, Carlsbad, CA, USA), 2 mmol/L L-glutamine, 0.2 mmol/L myoinositol, 0.1 mmol/L 2-mercaptoethanol, and 0.02 mmol/L folic acid (all Sigma, St. Louis, MO, USA) at 37°C in a 5% CO_2 incubator. Fresh medium was replaced every 2 d; cells reached confluence at 5 d of culture.

Cell proliferation assay

Cell viability was determined by trypsin blue exclusion. Here, NK92 cells were plated at 10^5 cells/well in 6-well plates. The cells were infected with bacteria at 100:1, 250:1, and 500:1 multiplicity of infection (MOI); control cells received phosphate-buffered saline (PBS, pH 7.4). After 24 and 48 h of culture, cell viabilities were assessed.

Animal experiments

C57BL/6 mice (male, 6-wk-of-age) were purchased from Orient Bio (Gyeonggi, Korea) and housed in pathogen-free facilities maintained at 20°C with 50% relative humidity and a 12-h light:dark cycle. All mice had *ad libitum* access to standard rodent chow and filtered water. The mice were acclimated for 1 wk and randomly divided into four groups (5 mice *per* group): Control, anti-control (antibiotics only), PBS (PBS after antibiotics), and FN (*F. nucleatum* after antibiotics). All mice (except control) were treated with a cocktail of broad-spectrum antibiotics (0.2 g/L ampicillin, 0.1 g/L vancomycin, 0.2 g/L neomycin, and 0.2 g/L metronidazole) in their drinking water for 3 wk to establish flora-deficient status. After 3 wk, the mice were administered 10^9 CFU *F. nucleatum* or the same dose of PBS by oral gavage every 2 d for 6 wk. At the end of the experiments, mice were anaesthetized with chloroform and euthanized by CO_2 gas to permit collection of biomaterials. All experimental procedures were performed with the approval of the Animal Experimentation Ethics Committee of Kosin University.

F. nucleatum quantification

Stool samples were collected from all mice once a week to assess the flora-deficient status and to compare the levels of total bacteria and *F. nucleatum*. For this, total DNA was isolated using the GeneAll Exgene Stool DNA mini kit (GeneAll Biotechnology Co. Seoul, Korea) according to the manufacturer's instructions. After quantification

and confirmation of purity using the Exgene Stool DNA mini kit (GeneAll, Seoul, Korea), an aliquot of total DNA (1 mg) was used as a template to be mixed with TB Green Premix Ex Taq II (Takara Bio, Shiga, Japan). The primers used for amplification were: *F. nucleatum* 5′-CGAGGAACCTTACCAGCGTT-3′ (F) and 5′-CCCAACATCT-CACGACACGA-3′ (R); total bacteria 5′-GTGSTGCAYGGYTGTCGTCA-3′ (F) and 5′-ACGTCRTCCMCACCTTCCTC-3′ (R); and GAPDH 5′-TGGCCTTCCGTGTTCCTAC-3′ (F) and 5′-GAG-TTGCTGTTGAAGTCGCA-3′ (R). All reactions were performed in triplicate in a 7300 Real-time polymerase chain reaction (PCR) system (Applied Biosystems, Waltham, MA, USA).

Assay of ex vivo NK cell activity

At necropsy, whole blood was collected from the heart and placed in sodium heparin-coated tubes for subsequent isolation of plasma. NK cell activity was then assessed (in triplicate) using a Murine NK activity kit (ATGen Co. Ltd., Seoul, Korea) according to the manufacturer's instructions. The absorbance generated for each sample reflected the relative amount of NK cell activity in the sample.

Immunohistochemical analyses of intestinal CD3 and NKp46 cells

Murine colon tissues were collected and placed in 10% buffered formalin overnight for fixing. Sections 4-μm thick were then prepared for immunohistochemical (IHC) staining using an automated immunostainer (Bond-MAX, Leica, Buffalo Grove, IL, USA). Antibodies against mouse CD3 (rabbit polyclonal, Dako, Agilent Technologies, city, state) and NKp46 (rabbit polyclonal, R&D Systems, Minneapolis, MN, USA) were used. Stained samples were then evaluated by light microscopy and immuno-activity was assessed. The $CD3^+$ or $NKp46^+$ cells were counted in five different fields (total = 500 counted cells) under 400 × magnification; cell counts were then converted to percentages for data presentation.

Biochemical assays

Plasma levels of pro-inflammatory interleukin (IL)-6, -1β, -18, and -12p70, and tumor necrosis factor (TNF)-α were measured (in duplicate) using commercial ELISA kits (Mouse IL-6 Uncoated ELISA, Mouse IL-18 Platinum ELISA, Mouse IL-1β Uncoated ELISA, Mouse IL-12p70 Platinum ELISA and Mouse TNF-α Uncoated ELISA; all Invitrogen). All experimental steps were performed at room temperature and in duplicate. Samples were quantified by spectrophotometry at 450 nm. The sensitivity levels of the kits were 4-500 pg/mL for IL-6, 8-1000 pg/mL for IL-1β, 31.2-2000 pg/mL for IL-18, 15.6-1000 pg/mL for IL-12p70, and 8-1000 pg/mL for TNF-α.

Statistical analysis

Each experiment was repeated at least three times. Data are expressed as means ± SD from each independent experiment. A Student's *t*-test was performed for continuous variables; outcomes with $P \leq 0.05$ were considered statistically significant. Statistical analyses were performed using SPSS Statistics software (v. 23.0, IBM, Armonk, NY, USA).

RESULTS

Cytotoxic effect of F. nucleatum, P. anaerobius, and P. micra on NK cells in vitro

The effects of various gut microbiota species on NK cell survival were first assessed. Here, *F. nucleatum*, *P. anaerobius,* and *P. micra* -all known to be associated with CRC- were cultured with NK cells for 24 or 48 h. To identify optimal amounts of gut microbiome species affecting NK cells, experiments with each were carried out at 100:1, 250:1, or 500:1 MOI. As shown in Figure 1, NK cell counts were decreased in co-cultures with *F. nucleatum*, *P. anaerobius,* and *P. micra.* The results were remarkable in co-cultures at both 24 and 48 h; however, *F. nucleatum* most markedly decreased NK cell counts after 48 h. Therefore, *F. nucleatum* was selected for further study here as the representative microbiome species for use in subsequent *in vivo* studies.

NK cell activity decreased in mice treated with F. nucleatum

To test whether *F. nucleatum* could reduce the activity of NK cells in mice, the animals were divided into four groups (5 mice *per* group): Control, anti-control, PBS and FN (Figure 2A). To establish flora-deficient status, mice were treated with a cocktail of broad-spectrum antibiotics for 3 wk. Confirmation of flora-deficiency was then

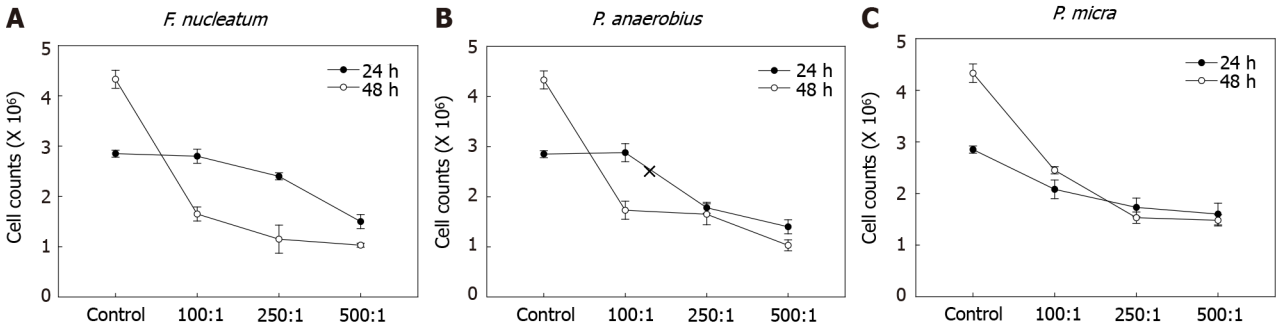


Figure 1 Effects of *Fusobacterium nucleatum*, *Peptostreptococcus anaerobius*, and *Parvimonas micra* on natural killer cells *in vitro*. Natural killer 92 cells were cultured for 24 or 48 h with each organism at a multiplicity of infection of 100:1, 250:1 or 500:1. Post-incubation, viability in the cultures was assessed using trypan blue staining. A: *Fusobacterium nucleatum*; B: *Peptostreptococcus anaerobius*; C: *Parvimonas micra*. *F. nucleatum*: *Fusobacterium nucleatum*; *P. anaerobius*: *Peptostreptococcus anaerobius*; *P. micra*: *Parvimonas micra*.

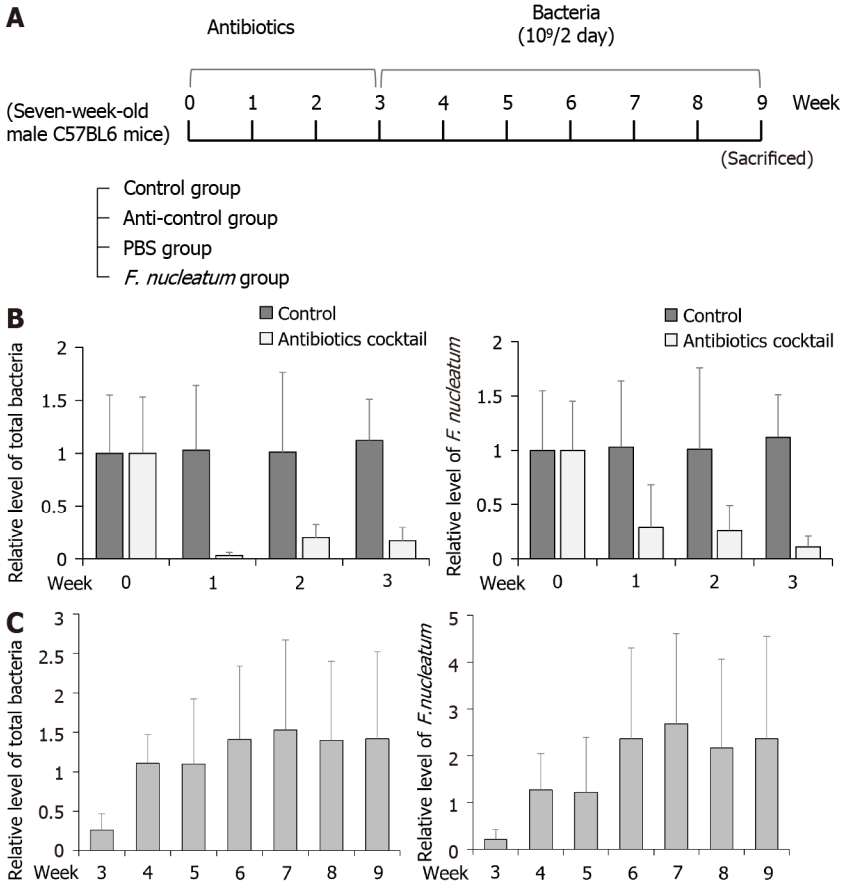


Figure 2 Experimental design. A: Mice were divided into four goups: Control, anti-control (antibiotics only), phosphate-buffered saline (PBS) (PBS after antibiotics), and *Fusobacterium nucleatum* (*F. nucleatum*) (*F. nucleatum* after antibiotics). The mice were treated with broad-spectrum antibiotics for 3 wk (except control), *F. nucleatum* was introduced to mice every 2 d for 6 wk by oral gavage; B: After treatments, the levels of total gut bacteria (left) and *F. nucleatum* (right) were evaluated; C: After administration of *F. nucleatum*, levels of total bacteria (left) and *F. nucleatum* (right) in mice were again evaluated. Data are expressed as means ± SD of three independent experiments. PBS: Phosphate-buffered saline.

assessed by quantitative PCR analyses of stool samples; the data confirmed that the levels of total bacteria and *F. nucleatum* were decreased (Figure 2B). At that point, *F. nucleatum* was re-introduced by oral gavage to the flora-deficient mice every 2 d for 6 wk. Analyses of their stools showed that levels of total bacteria and *F. nucleatum* gradually increased and then attained a plateau (Figure 2C).

The body weights of the mice were checked every week and compared. The weights of PBS and FN mice were lower compared to those of the control and anti-control groups after oral gavage; however, the differences were not significant (Figure 3A). At sacrifice (*i.e.*, after 6 wk of gavage treatments), the mice were euthanized and the entire colon of each was isolated, longitudinally-opened, and examined. No significant

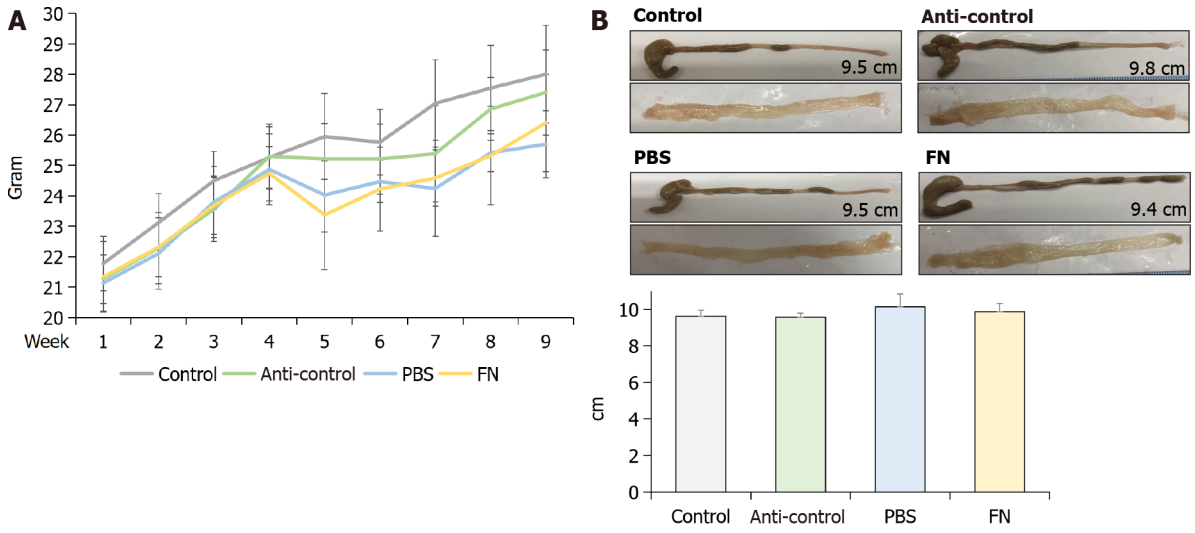


Figure 3 Body weight, colon length, and mucosal surface among the experimental groups. A: Weights of mice; B: Colon length and mucosal surface. PBS: Phosphate-buffered saline; FN: *F. nucleatum*.

differences in colonic length or colonic mucosal surface were found between the groups (Figure 3B).

To evaluate the activity of NK cells *in situ*, levels of interferon-gamma (IFN-γ) in the blood of each mouse was evaluated. The data indicated that the activity of NK cells in the FN group was significantly lower than in the control, anti-control, and PBS mice (Figure 4).

Local NK cell numbers in the gut decreased in mice treated with F. nucleatum

Using IHC staining, the intestinal tract of the mice was evaluated for the presence of NK cells. The data indicate that the levels of $CD3^+$ cells were significantly lower in the anti-control, PBS, and FN mice than in the control mice (Figure 5). The levels of $NKp46^+$ cells were lower in the FN group than in the control group, although the difference was not significant.

F. nucleatum treatments resulted in increased plasma pro-inflammatory cytokine levels

As shown in Figure 6, plasma levels of select pro-inflammatory cytokines were also evaluated at the end of the treatment regimens. The data indicated that circulating levels of IL-1β and TNF-α in the FN mice were significantly higher than in the control mice; values in the anti-control and PBS groups were not significantly higher than in the controls. Plasma levels of IL-6, IL-18, and IL-12p70 in the FN mice were not significantly higher than in the control. In addition, the values in the anti-control and PBS groups were not significantly changed compared to the control group.

DISCUSSION

Emerging evidence has shown a role for the gut microbiome, especially *F. nucleatum,* in cancer biology. Although *F. nucleatum,* a common bacterial resident in the oral cavity, has been shown to be involved in the proliferation of CRC cells and tumor development, data on how the Fusobacteria engage in the development of CRC are lacking. This study showed that a high abundance of *F. nucleatum* in the gastrointestinal tract reduced the activity of NK cells and that such outcomes might be associated with a concurrent increase in circulating levels of some pro-inflammatory cytokines, including IL-1β and TNF-α in these hosts. These findings suggest that increases in *F. nucleatum* in the gastrointestinal tract could be a factor in disrupting how the immune system prevents development of CRC.

The importance of the gut microbiome in immunity/inflammation has been more clearly elucidated in the past two decades. In a healthy state, the host immune system responds to intestinal microbiome strictly confined to the mucosal surface[18]. The mucus layer, epithelial cells, immunoglobulin A, dendritic cells, and T-cells comprise the mucosal barrier; these prevent the exposure and passage of gut microbiome to the

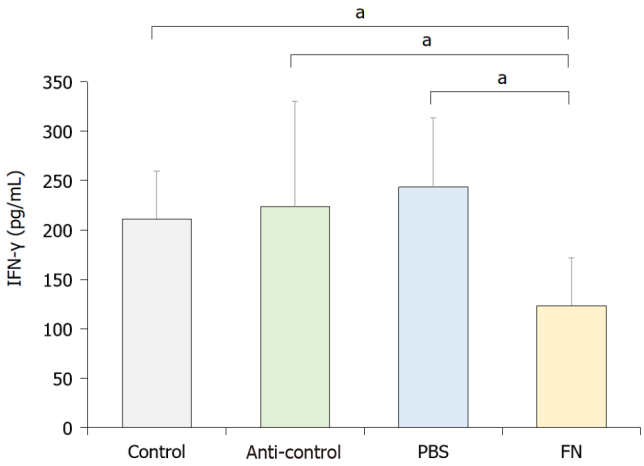


Figure 4 Blood levels of interferon-gamma. To indirectly evaluate the activity of natural killer cells, whole blood from each mouse was collected at necropsy and levels of interferon-gamma evaluated by ELISA. Data are expressed as means ± SD of three independent experiments. Value significantly different at $^{a}P < 0.05$. IFN-γ: Interferon-gamma; PBS: Phosphate-buffered saline; FN: *F. nucleatum*.

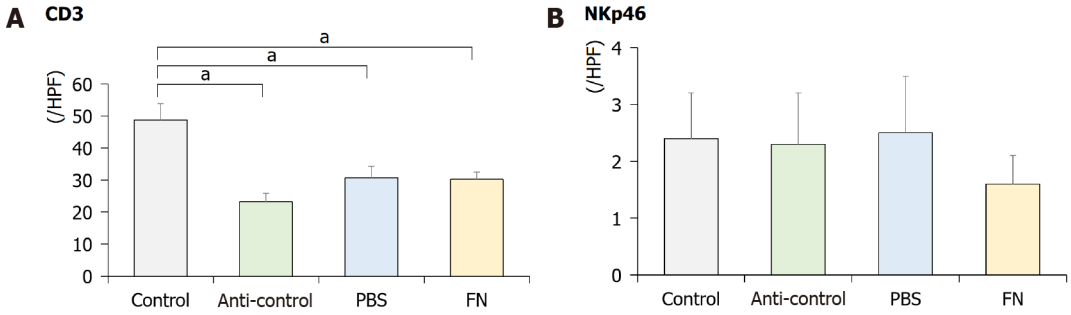


Figure 5 Intestinal levels of CD3 and NKp46 cells. A: $CD3^{+}$ cells; B: $NKp46^{+}$ cells. Data are expressed as means ± SD of three independent experiments. Value significantly different at $^{a}P < 0.05$. NK: Natural killer; PBS: Phosphate-buffered saline; FN: *F. nucleatum*.

gut-associated lymphoid tissue[19]. When the maintenance of tissue homeostasis is destroyed, translocating bacteria into the mucosal tissue causes a local (and systemic) effect on inflammatory cells, *i.e.*, tissue inflammatory monocytes can respond to microbial-derived ligands by producing prostaglandin E_2 which limits neutrophil activation and tissue damage[20].

F. nucleatum, whose presence has been proposed to correlate with CRC, can induce inflammation and suppress host immunity. There is increased evidence that *F. nucleatum* modulates immune cells in the CRC microenvironment and promotes intestinal tumorigenesis[21]. *F. nucleatum* can also stimulate autophagic pathways and alter chemotherapeutic responses of patients[22]. The current study focused on the impact of *F. nucleatum* upon gastrointestinal tract immune cells, especially NK cells. This study found that *F. nucleatum* could reduce local NK cell counts and decrease the activity of NK cells in the gut. Normally, NK cell activity is controlled by a balance of various signals that are delivered to the cells *via* inhibitory and activating NK cell receptors[23]. One study revealed that the ability of NK cells to kill tumors was inhibited by hemagglutinating *F. nucleatum* strains and that *F. nucleatum* inhibited immune cell activity by specifically targeting the inhibitory receptor TIGIT *via* the Fap2 protein[8]. Although the present study did not investigate the detailed mechanism for the observed inhibitory effects of *F. nucleatum* on the activity of NK cells in mice, the findings suggest that the gut microbiome could play an inhibitory role.

NK cells usually present as $CD56^{+}CD3^{-}$ cells and can be categorized into $CD3^{-}CD56^{dim}CD16^{+}$ and $CD3^{-}CD56^{bright}CD16^{-}$ cell classes. $CD56^{dim}$ cells appear to have more cytotoxic activity, while $CD56^{bright}$ cells produce high levels of cytokines including IFN-γ and TNF-α[24]. The activation and inhibition of NK cells are induced by an interplay among the cells' activating and inhibitory receptors[25]. NKp46 is expressed on NK cells in the tumor bed and is a major determinant of NK cell function[26]. In the current study, levels of $CD3^{+}$ cells significantly decreased after antibiotic treatment and $NKp46^{+}$ cells decreased after administration of *F. nucleatum*, although the difference

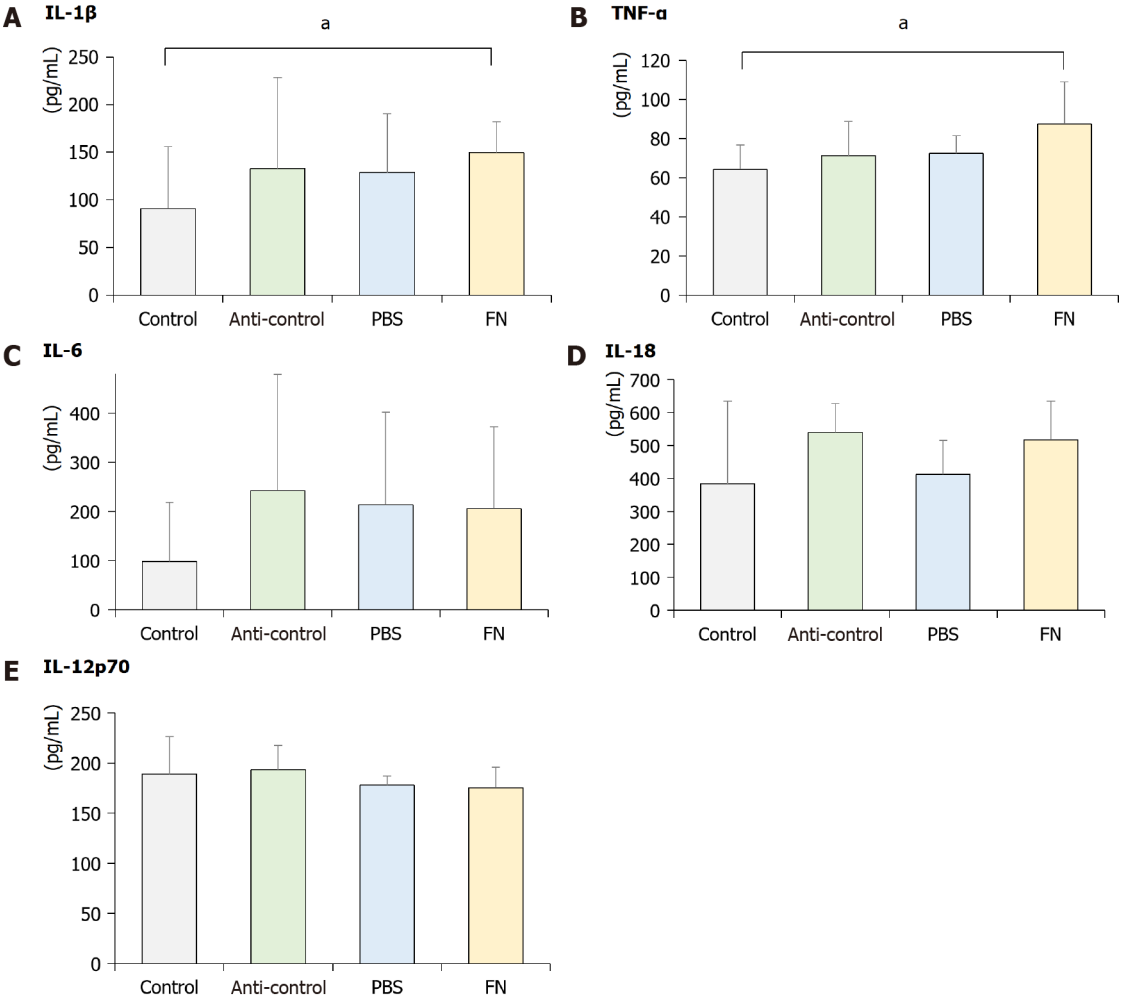


Figure 6 Plasma pro-inflammatory cytokine levels. A: Interleukin (IL)-1β; B: Tumor necrosis factor-α; C: IL-6; D: IL-18; E: IL-12p70. Data are expressed as means ± SD of three independent experiments. Value significantly different at $^{a}P < 0.05$. PBS: Phosphate-buffered saline; FN: *F. nucleatum*; IL: Interleukin; TNF-α: Tumor necrosis factor-α.

was not significant. Based on these results, it is thought that the microenvironment change after antibiotic treatment could affect these alterations, and the administration of *F. nucleatum* might decrease NK cell number.

Several inflammatory cytokines (including TNF-α, IL-6, IL-18, and IL-1β) are known to be involved in the initiation and progression of cancers[27], and could modulate the activation of NK cells. TNF-α can induce DNA damage through reactive oxygen and nitrogen species, IL-18 can induce the production of angiogenic and tumor growth stimulating factors, IL-6 can induce oxidative stress and promote early tumorigenesis, and IL-1β can promote neo-angiogenesis and evoke antiapoptotic signaling in tumor cells[28-30]. It is plausible that any changes in the expression in one or more of these could modulate tumor growth/survival *in situ* by also modulating the activation of NK cells[31]. For example, IL-12 can enhance the activity of NK cells and $CD8^{+}$ T-lymphocytes, and so is expected to impart anti-tumor activity[32,33]. In this study, it was seen that circulating levels of IL-1β and TNF-α (but not IL-6, IL-18, or IL-12p70) were significantly increased after administration of *F. nucleatum* to mice. Further studies are needed to determine if these changes directly/indirectly impact the activity of NK cells themselves in hosts.

This study has some limitations. First, whether decreased NK cell activity induces the development of CRC was not assessed. Further studies including the association between NK cell activity and CRC could strengthen the results. Second, the pro-inflammatory cytokine levels were not evaluated in NK92 cells and in colonic tissues treated with *F. nucleatum*. Therefore, changes in plasma pro-inflammatory cytokine levels in NK92 cells and in colonic tissues treated with *F. nucleatum* were not confirmed.

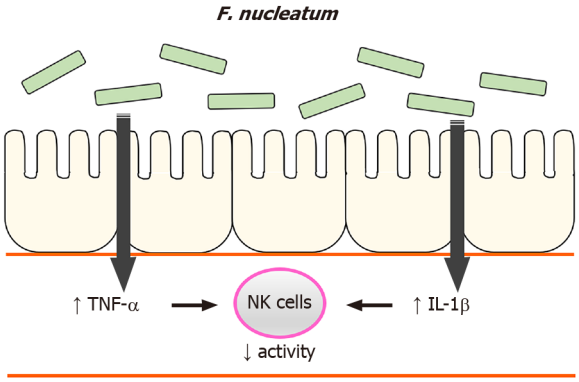


Figure 7 A possible mechanism. High levels of *F. nucleatum* in the gastrointestinal tract induce an increase in plasma pro-inflammatory cytokines, which reduces natural killer cell activity. IL: Interleukin; TNF-α: Tumor necrosis factor-α; NK: Natural killer.

CONCLUSION

In summary, the current study identified that high levels of *F. nucleatum* in the gastrointestinal tract reduced NK cell activity in mice and suggests insights on how NK cell activity was affected, *i.e.*, *F. nucleatum* induced systemic pro-inflammatory responses in the hosts. Based on these findings, we suggest a possible mechanism by which high levels of *F. nucleatum* in the gastrointestinal tract induce an increase in plasma pro-inflammatory cytokines, which reduces NK cell activity (Figure 7). Our findings illustrate another potential means by which gut microbiome can influence NK cells which, in turn could impact the development of CRC.

ARTICLE HIGHLIGHTS

Research background

Gut microbial dysbiosis contributes to the development and progression of colorectal cancer (CRC). Natural killer (NK) cells are involved in early defense mechanisms to kill infective pathogens and tumor cells by releasing chemokines and cytokines.

Research motivation

This study was designed to better understand the relationship between the gut microbiome and CRC.

Research objectives

The objective of this study was to identify associations between gastrointestinal tract *Fusobacterium nucleatum* (*F. nucleatum*) levels and NK cell activity.

Research methods

In vitro experiments were performed on NK cells treated with *F. nucleatum, Peptostreptococcus anaerobius,* and *Parvimonas micra* to identify the effects of gut microbiome species on NK cells. After 24 and 48 h of treatment, NK cell counts were measured. In parallel studies, C57BL/6 mice were given broad-spectrum antibiotics in their drinking water to reduce resident gut flora. After 3 wk, the mice received the various bacterial species or phosphate-buffered saline *via* oral gavage every 2 d for 6 wk. At the study end, blood samples were acquired to perform NK cell activity assessment and cytokine analysis. Intestinal tissues were collected and analyzed *via* immunohistochemistry (IHC).

Research results

After 3 wk of broad-spectrum antibiotic treatment, levels of total bacteria and *F. nucleatum* were markedly decreased in mice. *F. nucleatum* by gavage significantly decreased NK cell activity relative to the activities of cells from mice treated with antibiotics only and phosphate-buffered saline. The administration of *F. nucleatum* decreased the proportion of $NK46^+$ cells based on IHC staining and increased the production of interleukin-1β and tumor necrosis factor-α.

Research conclusions

High levels of *F. nucleatum* in the gastrointestinal tract reduced NK cell activity in mice, and the decrease in NK cell activity might be affected by increased pro-inflammatory cytokines after *F. nucleatum* treatment.

Research perspectives

Based on these findings, we suggest that increases in *F. nucleatum* in the gastrointestinal tract could be a factor in disrupting how the immune system prevents development of CRC.

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ORIGINAL ARTICLE

Retrospective Study

Poor performance of anti-mitochondrial antibodies for the diagnosis of primary biliary cholangitis in female Colombian patients: A single-center study

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Abstract

BACKGROUND

Primary biliary cholangitis (PBC) is a serious disease that causes significant morbidity. PBC is confirmed with liver biopsy but autoantibodies are frequently used as proxies for diagnosis. The performance of autoantibodies for the diagnosis of PBC seems to vary widely across populations.

AIM

To assess the diagnostic performance of several autoantibodies for the diagnosis of PBC in Latin American individuals.

METHODS

We studied 85 female adult Colombians, 43 cases with biopsy-confirmed PBC and 42 controls in whom a liver biopsy ruled out PBC. Plasma anti-mitochondrial antibodies (AMAs), anti-smooth muscle antibodies (ASMAs) and anti-nuclear antibodies (ANAs), as well as total immunoglobulin (Ig) M and IgG were determined using immunofluorescence or enzyme-linked immunosorbent assay in all study participants within 1 year of the biopsy. For all variables, values analyzed were those closest to the date of the biopsy. Patients with viral or alcoholic hepatitis were excluded.

Committee of the Universidad de los Andes.

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RESULTS

Mean age at diagnosis was 58.7 years for cases and 56.9 years for controls, and the body mass index was lower among cases. Most cases received ursodeoxycholic acid, while most controls received vitamin E. Sjögren syndrome and Hashimoto's thyroiditis were the most frequent autoimmune comorbidities of PBC. The prevalence of AMA positivity among PBC cases was unexpectedly low. The sensitivity and specificity values were respectively 44.2% and 76.2% for AMA, 74.4% and 38.1% for ANA, 14.0% and 73.8% for ASMA, 26.7% and 80.0% for IgG, and 57.1% and 85.7% for IgM. The combination of positive AMA plus positive IgM had 91% positive predictive value for PBC. Among AMA-negative cases, the most prevalent antibodies were ANA (87.5%). In all, 62% of AMA-positive and 84.6% of IgM-positive individuals had fibrosis in their biopsy.

CONCLUSION

AMA positivity was very low among female Latin American patients with PBC. The performance of all antibodies was quite limited. These results highlight the urgent need for better PBC biomarkers.

Key Words: Primary biliary cholangitis; Antibodies; Anti-mitochondrial antibodies; Latin America; Anti-smooth muscle antibodies

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Core Tip: Primary biliary cholangitis (PBC) is confirmed by liver biopsy, but autoantibodies are frequently employed as an indirect diagnostic method. We studied 85 female adult Latin American patients, 43 with biopsy-confirmed PBC and 42 in whom liver biopsy ruled out PBC. The prevalence of anti-mitochondrial antibodies among PBC cases was only 44.2%. Anti-nuclear antibodies and anti-smooth muscle antibodies had similarly low diagnostic performance. Eighty-two percent of immunoglobulin M-positive individuals had fibrosis in their biopsy. Among female Latin American patients with PBC, the performance of any individual antibody for PBC diagnosis was quite limited.

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INTRODUCTION

Primary biliary cholangitis (PBC) is a chronic autoimmune liver disease characterized by cholestasis, autoantibodies, and chronic small bile duct injury[1]. In PBC, immune-mediated portal inflammation leads to a group of histological hallmarks: biliary epithelial damage, ductopenia, and fibrosis[2]. The disease was previously known as "primary biliary cirrhosis," a term coined in 1950 but no longer in use because in fact most individuals are not cirrhotic at the time of diagnosis[3]. The population incidence of PBC is approximately 1 case per 100000 individuals per year, but there is significant geographic variation[4]. PBC is 10 times more common among women than men, and is frequently diagnosed between 40 and 50 years of age[5]. Patients with PBC may be asymptomatic, or may present with jaundice, fatigue, or pruritus[6].

The gold standard for PBC diagnosis is liver biopsy, but measurement of plasma autoantibodies is considered a key step of the initial approach[1]. There is a large repertoire of serologic tests with diagnostic potential for PBC including anti-mitochondrial antibodies (AMAs), anti-nuclear antibodies (ANAs), antibodies against smooth muscle (ASMA), titers of immunoglobulin (Ig) G and IgM, and more than 60 other immunoassays[7,8].

However, the performance of these tests varies widely across populations[4], and there is a critical lack of information about their diagnostic value in Hispanic patients. Within this context, the primary aim of our study was to evaluate various antibodies and antibody combinations for the diagnosis of PBC in Latin American individuals from Colombia.

MATERIALS AND METHODS

Participants

We retrospectively collected information from 86 participants, 44 patients with a biopsy confirmed diagnosis of PBC and 42 controls in whom the results of liver biopsy ruled out PBC. Inclusion criteria were age over 18 years, having a liver biopsy performed between 2009 and 2019 and availability of a measurement of circulating AMA, ASMA and ANA by immunofluorescence or enzyme-linked immunosorbent assay (ELISA), within 1 year of the index liver biopsy. Exclusion criteria were alcohol consumption equal to or greater than two drinks a day on average, or viral hepatitis (serology for hepatitis B and hepatitis C viruses were available at the time of biopsy for all participants). All PBC diagnoses were confirmed or ruled out by a qualified pathologist with experience in liver diseases. Controls had to have biopsies reported as normal, non-alcoholic fatty liver disease or other, non-cholestatic liver diseases.

The definitive sample consisted of 85 participants, after we excluded the only male PBC case in order to have a more homogeneous study group. We extracted from their medical history data on demographics, blood chemistry, liver function tests, clinical presentation, concomitant medications, and autoimmune comorbidities. The laboratory values considered were those closest to the date of the biopsy. All clinical records were anonymized prior to data extraction and analysis. The study database was secured and only two researchers had access to it (Gaete PV and Puerto-Baracaldo DK).

Sample size

We calculated sample size using the expression for diagnostic tests studies in which the expected prevalence of the condition of interest is known[9]. Concerning the results of AMA for diagnosis of PBC, if *FP* is the number of false positives, *TN* is the number of true negatives, *Z* is the value of the *Z* statistic for the 95th quantile of the standard normal distribution, *SP* is the minimum tolerable specificity (90%), *W* is the confidence interval width for the estimation of specificity obtained from the study (10%) and *P* is the expected prevalence of PBC among the included individuals (50%), the sample size for specificity (N_{sp}) is given by the following expression:

$$FP + TN = Z^2 \times SP \times (1 - SP)/W^2$$

$$N_{sp} = (FP + TN)/(1 - P)$$

This gave us a minimum sample size of 69 participants. Estimating an additional 20%, 86 participants were included.

Statistical analyses

Baseline characteristics were compared between cases and controls using *t*-tests for continuous variables and chi-squared tests for categorical variables. The sensitivity, specificity, and predictive values for PBC diagnosis were calculated for each antibody and their relevant combinations, considering liver biopsy as the gold standard. Additionally, we plotted receiver-operating characteristic (ROC) curves for titers of AMA, ASMA, and ANA. The association between antibody positivity and the presence of fibrosis was assessed using odds ratio (OR) calculated with univariate logistic regression. All statistical analyses were two-tailed and performed at a 5% significance level (alpha = 0.05) using IBM SPSS, version 20.0.

RESULTS

We studied 85 female adults, 43 with PBC and 42 controls. Mean age at diagnosis was 58.7 years for cases and 56.9 years for controls. Mean body mass index was lower among cases ($P = 0.001$), perhaps because a suspicion of nonalcoholic steatohepatitis was the indication for liver biopsy in many controls (Table 1). As expected, alkaline phosphatase was significantly higher among cases, but markers of hepatocellular damage were similar in both groups. Pruritus was the most common symptom in

Table 1 Characteristics of study participants

	Controls	Cases	*P* value
Age at diagnosis	58.7 ± 11.4	56.9 ± 10.2	0.44
Body mass index	26.8 ± 3.4	23.8 ± 2.4	0.001
Total cholesterol (mg/dL)	221 ± 52.5	221 ± 45.3	0.98
LDL cholesterol (mg/dL)	125 ± 40.2	124.8 ± 41.9	0.99
HDL cholesterol (mg/dL)	54.1 ± 28.7	58.5 ± 10.6	0.48
Triglycerides (mg/dL)	157 ± 64.6	134 ± 54.8	0.16
Fold increment of ALKP above ULN	1.82 ± 1.55	2.84 ± 2.06	0.013
Fold increment of ALT above ULN	2.27 ± 2.94	2.23 ± 1.97	0.94
Fold increment of AST above ULN	2.08 ± 3.37	2.06 ± 1.6	0.97
Fold increment of GGT above ULN	4.43 ± 3.57	6.74 ± 6.78	0.087
Bilirubin (mg/dL)	0.68 ± 0.42	1.5 ± 1.9	0.007
Albumin (g/L)	4.09 ± 0.32	4.05 ± 0.36	0.75
International normalized ratio	0.99 ± 0.09	1.0 ± 0.13	0.56
Ascites, *n* (%)	2 (4.8)	3 (7)	0.66
Pruritus, *n* (%)	12 (28.6)	20 (46.5)	0.08
Degree of fibrosis, *n* (%)[1]			
0	21 (50)	18 (41.9)	0.031
1	14 (33.3)	8 (18.6)	
2	1 (2.4)	11 (25.6)	
3	4 (9.5)	5 (11.6)	
4	2 (4.8)	1 (2.3)	
Pattern of anti-nuclear antibodies, *n* (%)			
Peripheral	0 (0)	1 (2.3)	0.37
Homogeneous	0 (0)	0 (0)	
Gross speckled	0 (0)	0 (0)	
Fine speckled	0 (0)	2 (4.7)	
Centromeric	9 (21.4)	12 (27.9)	
Nucleolar	2 (4.8)	2 (4.7)	
Laminar	0 (0)	0 (0)	
Cytoplasmic	6 (14.3)	2 (4.7)	
Medications at diagnosis, *n* (%)			
Ursodeoxycholic acid	16 (38.1)	37 (86.0)	< 0.001
Vitamin E	15 (35.7)	3 (7.0)	0.001
Statins	6 (14.3)	2 (4.7)	0.13
Antidiabetics	5 (11.9)	1 (2.3)	0.085
Immunosuppressors	11 (26.2)	13 (30.2)	0.68
Levothyroxine	15 (35.7)	15 (34.9)	0.94
Autoimmune comorbidities, *n* (%)			
Sjögren syndrome	4 (9.5)	9 (20.9)	0.14
Limited systemic sclerosis	2 (4.8)	4 (9.3)	0.41
Diffuse systemic sclerosis	4 (9.5)	2 (4.7)	0.381

Hashimoto´s thyroiditis	5 (11.9)	6 (14)	0.78
Graves´ disease	2 (4.8)	0 (0)	0.15
Systemic lupus erythematosus	1 (2.4)	0 (0)	0.31
Inflammatory bowel disease	0 (0)	0 (0)	-
Rheumatoid arthritis	1 (2.4)	4 (9.3)	0.178
Hemolytic autoimmune anemia	1 (2.4)	0 (0)	0.31
Autoimmune thrombocytopenic purpura	1 (2.4)	0 (0)	0.31

Data are mean ± SD unless stated otherwise.
[1]According to the Batts Ludwig classification[33].
ALKP: Alkaline phosphatase; ALT: Alanine amino transferase; AST: Aspartate amino transferase; GGT: Gamma-glutamyl transferase; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; ULN: Upper limit of normal.

participants with PBC. Fibrosis in stages II-IV of the Batts-Ludwig classification was much more common in cases (39.5% *vs* 16.7%). In both groups, the most frequent pattern of ANA was centromeric. Most cases received ursodeoxycholic acid (UDCA) while most controls received vitamin E. Sjögren syndrome and Hashimoto's thyroiditis were the most frequent autoimmune comorbidities of PBC. The most common histological findings in our PBC cases were destructive, non-suppurative cholangitis (39.5%), florid duct lesions (27.9%), interphase hepatitis in biopsy (23.3%) and granulomatous cholangitis (14.0%).

The prevalence of positivity for AMA in cases was surprisingly low, only 44.2%. Among participants with positive AMA, the most prevalent antibody was IgM, present in 91.7% of cases and 33.3% of controls. Meanwhile, among participants with negative AMA, the most prevalent antibodies were ANA, present in 87.5% of cases and 59.4% of controls. None of the AMA-negative controls had a positive IgM, while 11.1% of the AMA-negative cases did (Figure 1).

The diagnostic performance of AMA in our sample was lower than expected, with a sensitivity of 44.2% and a specificity of 76.2%. ANA showed better sensitivity (74.4%) but low specificity (38.1%). IgM had an acceptable balance between sensitivity (57.1%) and specificity (85.7%). Of note, the combination of positive AMA and positive ASMA had 100% specificity for confirmation of PBC. The combination of negative AMA and positive IgM exhibited 100% specificity and positive predictive value (Table 2).

Fibrosis was more frequent among participants with positive AMA or IgM (Figure 2): Sixty-two percent of AMA-positive and 84.6% of IgM-positive individuals had any degree of fibrosis in biopsy. There was an equal proportion of persons with and without fibrosis among AMA-negative participants, while fibrosis was unusual (33.3%) when IgM was negative. Among cases only, the proportion with fibrosis was 63% for AMA-positive and 54% for AMA-negative individuals. The OR for the association between positive AMA and any fibrosis was not significant [OR:1.63, 95% confidence interval (CI): 0.67-4.09], while the association between positive IgM and fibrosis was strongly positive (OR:11.0, 95%CI: 1.73-69.9). Fibrosis was also differently associated with the predominant pattern in liver biopsy. The proportion of individuals with fibrosis was 80% for patients with lymphocytic infiltrate, 70.6% for patients with destructive non-suppurative cholangitis, 66.6% for patients with granulomatous cholangitis or florid lesions and 65% for patients with interphase hepatitis.

In separate exploratory ROC analyses of the plain reported titers of AMA and ANA, the best cutoff for AMA titers was 1:50, showing a sensitivity of 45.2%, a specificity of 81.1% and an area under the curve (C-statistic) of 66%. Meanwhile, the best cutoff for ANA titers was 1:120, with a sensitivity of 71.8%, a specificity of 52.5% and a C-statistic of 64.1%.

We performed a sensitivity analysis in which PBC status was defined as having a pathology diagnosis of PBC plus elevation of alkaline phosphatase. The results were very similar to those obtained with PBC status defined only by biopsy results, except for a slightly better negative predictive value for AMA (63.6 *vs* 57.1%) and slightly better sensitivity for IgM (63.2% *vs* 57.1%).

Table 2 Sensitivity, specificity and predictive values of each antibody or antibody combination, for the diagnosis of primary biliary cholangitis

Antibody or antibody combination	Sensitivity	Specificity	PPV	NPV
AMA (+)	44.2	76.2	65.5	57.1
ANA (+)	74.4	38.1	55.2	59.3
ASMA (+)	14.0	73.8	35.3	45.6
IgG (+)	26.7	80.0	66.7	42.1
IgM (+)	57.1	85.7	92.3	40.0
AMA (+), ANA (+)	25.6	83.3	61.1	52.2
AMA (+), ANA (-)	18.6	92.9	72.7	52.7
AMA (-), ANA (+)	48.8	54.8	52.5	51.1
AMA (+), ASMA (+)	7.0	100	100	51.2
AMA (+), ASMA (-)	37.2	76.2	61.5	54.2
AMA (-), ASMA (+)	7.0	73.8	21.4	43.7
ANA (+), ASMA (+)	9.3	83.3	36.4	47.3
ANA (+) , ASMA (-)	65.1	54.8	59.6	60.5
ANA (-), ASMA (+)	4.7	90.5	33.3	48.1
AMA (+), IgG (+)	13.3	90.0	66.7	40.9
AMA (+), IgG (-)	46.7	70.0	70.0	46.7
AMA (-), IgG (+)	13.3	90.0	66.7	40.9
AMA (+), IgM (+)	52.4	85.7	91.7	37.5
AMA (+), IgM (-)	4.8	71.4	33.3	20.0
AMA (-), IgM (+)	4.8	100	100	25.9

AMA: Anti-mitochondrial antibodies; ANAs: Anti-nuclear antibodies; ASMA: Anti-smooth muscle antibodies; NPV: Negative predictive value; IgG: Immunoglobulin G; IgM: Immunoglobulin M; PPV: Positive predictive value.

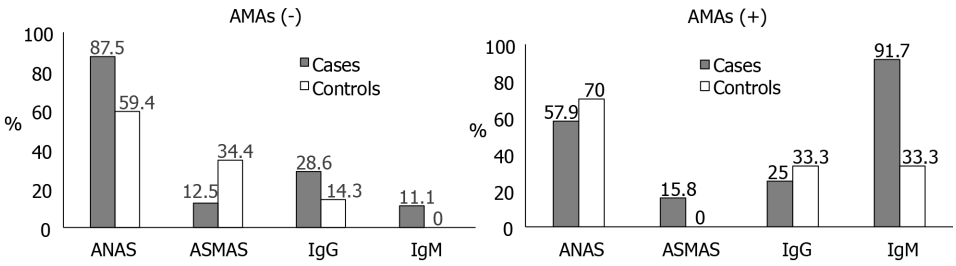


Figure 1 Prevalence of positivity for other antibodies, among participants with negative (left) or positive (right) anti-mitochondrial antibodies. Case/control status was defined by liver biopsy. AMAs: Anti-mitochondrial antibodies; ANAs: Anti-nuclear antibodies; ASMAs: Anti-smooth muscle antibodies; IgG: Positive immunoglobulin G, defined as a plasma concentration greater than the upper limit of the reference value; IgM: Positive immunoglobulin M, defined as a plasma concentration greater than the upper limit of the reference value.

DISCUSSION

In this cross-sectional study, we assessed the performance of different autoimmunity markers for the diagnosis of biopsy confirmed PBC in the context of female Latin American patients. We were surprised to find that the majority of PBC cases were AMA-negative, and that no individual antibody or antibody pair provided a distinctly superior combination of sensitivity and specificity. Our results also suggested that IgM positivity can be a tool for the diagnosis of AMA-negative PBC and that IgM may serve as a marker of liver fibrosis. The results also show that simultaneous positivity for AMA and ASMA was a relevant confirmatory finding for PBC.

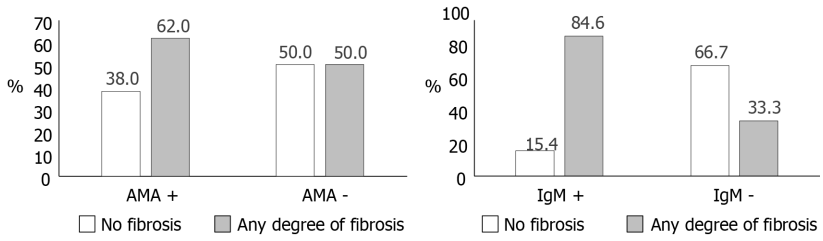


Figure 2 Distribution of the degree of fibrosis according to positivity for anti-mitochondrial antibodies (left) or immunoglobulin M (right). Odds ratio for the association between positive anti-mitochondrial antibodies and any fibrosis: 1.63 (95% confidence interval: 0.67-4.09), odds ratio for the association between positive immunoglobulin M and any fibrosis: 11.0 (95% confidence interval: 1.73-69.9). AMAs: Anti-mitochondrial antibodies; IgM: Immunoglobulin M.

The clinical characteristics of our Hispanic patients regarding age, sex, symptoms, and liver function tests were in accordance with prior reports of PBC patients worldwide and in other Latin American countries[10-15]. Sjögren syndrome and Hashimoto´s thyroiditis were the most prevalent autoimmune comorbidities in our sample, as has been reported in Mexican[13] and Italian[16] PBC cohorts. The frequent coexistence of PBC and Sjögren syndrome could be explained by the exposure of self-antigens in biliary and salivary epithelial cells, as a result of apoptosis (secondary to infections or xenobiotics), leading to autoimmune epithelitis[17]. Among PBC patients, Hashimoto's thyroiditis is the most frequently reported thyroid disorder, followed by Graves´ disease[16]. However, a study comparing two European cohorts found that thyroid disease does not influence the rate of complications nor the natural history of PBC[18]. In general, the coexistence of thyroid and liver disease is usual, as thyroid disturbances are also frequent in primary sclerosing cholangitis and non-alcoholic fatty liver disease[19]. These findings highlight the need for autoimmune disease screening in Latin American patients with PBC.

The performance of AMA, ANA, ASMA, IgM, and IgG has been evaluated before, albeit seldom in Hispanics or Latin Americans. In a Mexican cohort of 78 PBC patients, 94.8% had positive AMA, 70.5% positive ANA and 8% positive ASMA[13]. This is in stark contrast to the rate of AMA positivity of cases in our study, only 44.2%. A meta-analysis of 24 studies (none of them from Latin America), reported 84.5% sensitivity and 97.8% specificity for AMA, also quite different from our results[20]. A meta-analysis of 11 studies from Asia, Europe, and North America reported 27% sensitivity and 98% specificity for ANA in AMA-negative patients. We, in turn, found a high rate of ANA positivity among AMA-negative cases (87%)[21]. In a group of AMA-negative PBC patients from the Mayo Clinic, there was a 21% rate of ASMA positivity[22], similar to the 12.5% we found. These findings suggest the presence of factors in our population that influence the diagnostic performance of the classic autoantibodies for histologically confirmed PBC diagnosis. One of such factors may be polymorphisms in genes for proteins involved in antigen presentation at the bile ducts. It has been demonstrated that cholangiocytes express not only MHC class I molecules, but also surface markers found on antigen presenting cells including MHC class II and co-stimulatory molecules (CD80, CD86, CD40)[23]. Since the distribution of HLA alleles differs substantially across Latin America[24], it is conceivable that individuals from certain populations are better presenters of ductal self-antigens, and elicit a stronger humoral self-immunity in the context of PBC. This may constitute a potential explanation for the stark contrast between our results and those from the prior Mexican study.

The previously mentioned Mexican study also described Sjögren syndrome as the most common autoimmune comorbidity of PBC. In contrast to our results, they reported a high prevalence of AMA positivity among PBC cases, evidencing variability even within Latino populations. A cross-sectional study in the US found that Hispanic patients with PBC had a particular clinical picture, characterized by a reduced clinical response to UDCA[25].

Even though AMA are the antibodies classically related to PBC, there is evidence that most patients with positive AMA do not have PBC findings in liver biopsy[26,27]. More than half of PBC cases in our sample were AMA-negative, much higher than in prior reports (5%-18%)[28]. Evidence that clinical characteristics or response to treatment is different in AMA-negative patients has been inconsistent[29]. Nonetheless, it is clear that diagnosis of these patients tends to be delayed, treatment is usually initiated later and complications like fibrosis or liver transplantation are more frequent[22,30,31]. A study found that seven of nine patients with AMA-negative PBC

had the same immunohistochemistry findings as AMA-positive patients, supporting the idea that these subgroups have indeed the same disease[32]. One potential limitation of the use of AMAs in regular clinical practice is the availability of different techniques for their measurement, depending on the context (immunofluorescence *vs* ELISA), which may yield different diagnostic performance.

The utility of IgM in the study of PBC is being explored intensively, especially in the context of AMA-negative patients[33]. In our sample, the combination of negative AMA and positive IgM was highly specific for PBC. We also found that > 90% of AMA-positive cases had positive IgM. All things considered, IgM may be understood not only as a consequence of cholestasis, but also as a sign of alarm to look for fibrosis in PBC patients, even when AMA are negative. The mechanism underlying the high frequency of IgM positivity in AMA-positive PBC patients may involve polymorphisms in the gene for toll-like receptor 9[34].

Strengths of the study include a homogeneous sample of well-defined patients and controls of the same sex, the availability of relevant information on comorbidities and current treatments, the fact that PBC status was confirmed by biopsy and the uniqueness of the patient population ethnicity. This study is a valuable source of demographic and diagnostic information for Hispanic/Latino patients with suspected PBC.

The main limitations are the cross-sectional nature of the study, which does not allow drawing definitive conclusions about causal associations, and its relatively modest sample size, although it was appropriate for the diagnostic evaluation of the tests analyzed. Future studies are needed to describe the time-course and severity of the disease, in order to better approach PBC diagnosis and treatment in these populations.

CONCLUSION

In summary, this study showed that a large proportion of PBC cases in Latin American patients can be AMA-negative, and that IgM can be a useful marker of fibrosis, in addition to cholestasis. For all these reasons, it is urgent to find a good marker for AMA-negative PBC, especially for Hispanic/Latino populations.

ARTICLE HIGHLIGHTS

Research background

Primary biliary cholangitis (PBC) is a rare but serious and severely limiting autoimmune liver disease. Diagnosis of PBC is complicated by the different diagnostic performance of different autoantibodies in different populations.

Research motivation

There are limited data on the performance of autoantibodies for PBC diagnosis among Hispanic/Latino populations.

Research objectives

To assess the diagnostic performance of anti-mitochondrial antibodies (AMAs), anti-nuclear antibodies (ANAs), anti-smooth muscle antibodies (ASMAs), plasma immunoglobulin (Ig) M and plasma IgG for the diagnosis of histologically-confirmed PBC among female Colombian patients.

Research methods

We studied 43 PBC cases and 42 controls in whom PBC was ruled out. All antibodies were measured using immunofluorescence or enzyme-linked immunosorbent assay within 1 year of the index biopsy. A sensitivity analysis was performed using pathology + elevated alkaline phosphatase to define case-control status. Patients with viral or alcoholic hepatitis were excluded.

Research results

The sensitivity of ANA was only 44.2%, specificity was 76.2%. No individual antibody or antibody combination displayed an acceptable combination of sensitivity and specificity, but the combination of positive AMA and positive ASMA had a very high

specificity. IgM had particularly high specificity and positive predictive value.

Research conclusions

We found a remarkably high prevalence of AMA-negative PBC among female Colombian patients. IgM served not only as a marker of PBC, but also as a marker of liver fibrosis.

Research perspectives

It is urgent to find a good marker for AMA-negative PBC, especially for Hispanic/Latino populations.

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ORIGINAL ARTICLE

Clinical Trials Study

Effects of permissive hypocaloric *vs* standard enteral feeding on gastrointestinal function and outcomes in sepsis

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Abstract

BACKGROUND

Intestinal mucosal barrier injury and gastrointestinal dysfunction are important causes of sepsis. However, few studies have investigated the effects of enteral underfeeding on gastrointestinal function in sepsis. Moreover, no consensus on goal enteral caloric intake has been reached in sepsis.

AIM

To investigate the effects of different goal caloric requirements of enteral nutrition on the gastrointestinal function and outcomes in the acute phase of sepsis.

METHODS

Patients were randomly assigned to receive 30% (defined as group A), 60% (group B), or 100% (group C) of goal caloric requirements of enteral nutrition in this prospective pilot clinical trial. The acute gastrointestinal injury (AGI) grades, incidence of feeding intolerance (FI), daily caloric intake, nutritional and inflammatory markers, and biomarkers of mucosal barrier function were collected during the first 7 d of enteral feeding. The clinical severity and outcome variables were also recorded.

RESULTS

A total of 54 septic patients were enrolled. The days to goal calorie of group C (2.55 ± 0.82) were significantly longer than those of group A (3.50 ± 1.51; $P = 0.046$) or B (4.85 ± 1.68; $P < 0.001$). The FI incidence of group C (16.5%) was higher than that of group A (5.0%) or B (8.7%) ($P = 0.009$). No difference in the incidence of FI symptoms was found between groups A and B. The serum levels of barrier

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function biomarkers of group B were significantly lower than those of group A ($P < 0.05$) on the 7th day of feeding. The prealbumin and IL-6 levels of group A were lower than those of group B ($P < 0.05$) on the 7th day of feeding. No significant differences in the clinical outcome variables or 28-d mortality were found among the three groups.

CONCLUSION

Early moderate enteral underfeeding (60% of goal requirements) could improve the intestinal barrier function and nutritional and inflammatory status without increasing the incidence of FI symptoms in sepsis. However, further large-scale prospective clinical trials and animal studies are required to test our findings. Moreover, the effects of different protein intake on gastrointestinal function and outcomes should also be investigated in future work.

Key Words: Enteral feeding; Enteral nutrition; Gastrointestinal function; Intestinal mucosal barrier; Sepsis

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Core Tip: Few studies have investigated the effects of enteral underfeeding on gastrointestinal function in sepsis. Moreover, no consensus on goal enteral caloric intake has been reached in sepsis. In this study, we investigated the effects of different goal caloric requirements (30%, 60%, and 100%) of enteral nutrition on the gastrointestinal (including intestinal mucosal barrier) function in the acute phase of sepsis. We found that early moderate enteral underfeeding (60% of goal requirements) could improve the intestinal barrier function and nutritional and inflammatory status without increasing the incidence of feeding intolerance symptoms in sepsis.

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INTRODUCTION

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection[1-3]. Although the latest guidelines recommend a series of treatment strategies for sepsis[1,2], the mortality of this critical illness is still approximately 20%-50% in adults[4,5]. As an essential treatment for sepsis, enteral nutrition (EN), especially early enteral nutrition (EEN), could improve immunologic imbalance and alleviate the intestinal barrier injury of patients in intensive care units (ICUs)[6-9]. Our previous clinical studies also confirmed that EEN could regulate the excessive immune response and improve the clinical severity of critically ill patients[10,11]. However, recent trials suggested that aggressive nutrition delivery may offer no benefit in the early stages of critical illness[12-17]. The TARGET trail showed that augmented energy delivery in the early phase of illness did not improve outcomes compared to standard EN[14]. The ESICM guidelines advise that EEN (within 24-48 h) should be started at a low dose and increased gradually if there are no contraindications[9]. The ESPEN guidelines also advise that the initiation of "early and progressive" EN should be only performed in sepsis without shock[18].

Until now, no consensus on goal enteral caloric intake has been reached in critically ill patients. The EDEN trial observed that initial trophic enteral feeding for up to 6 d (400 kcal/d), compared with full enteral feeding (1300 kcal/d), did not improve ventilator-free days, 60-d mortality, or infectious complications in patients with acute lung injury[19]. The PermiT Trial found that permissive enteral underfeeding was not associated with a lower mortality compared with standard feeding (46% ± 14% *vs* 71% ± 22% of goal caloric requirements) in critically ill adults[17]. Systematic reviews of clinical trials also reported that hypocaloric EN (15%-59% of caloric requirements) had

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no significantly different effects on morbidity and mortality in critically ill patients when compared with full-energy nutrition[13,15]. However, most of the previous studies were based on non-sepsis patients. Furthermore, few studies have investigated the effects of enteral underfeeding on gastrointestinal function in sepsis.

Among the organ dysfunction caused by sepsis, intestinal tract is one of the most vulnerable organs[4,5]. Accompanying by sepsis, intestinal epithelial cell damaged, mucosal permeability increased, intestinal flora translocated, and then further intestinal original infection developed[3,4]. Therefore, acute intestinal barrier injury and systemic infection are a vicious cycle in critical diseases, especially in sepsis. Accordingly, it is necessary to explore an optimal goal of enteral feeding to improve the acute intestinal injury of sepsis. In this study, we investigated the effects of different goal caloric requirements (30%, 60%, and 100%) of EN on the gastrointestinal (including intestinal mucosal barrier) function in the acute phase of sepsis.

MATERIALS AND METHODS

Study design

This was a single-center, prospective, randomized clinical trial. Patients were randomly assigned to receive 30%, 60%, or 100% of goal caloric requirements of EN after enrollment. We used the complete randomization, which was based on remainder grouping method[10]. After creating random numbers with a computer, the grouping method (divided by three) was implemented to determine which group the patients would be allocated into. The intervention allocation was concealed before a patient was enrolled in this study, and patients would not be excluded from the study after intervention allocation was unblinded. The main treatment difference of the three groups was the goal calorie of enteral feeding, therefore, it was improbable to blind treating clinicians to the intervention allocations. The enrolled patients were blind to the intervention allocations. Hence, this is a single-blind randomized trial. The sample size calculation was performed with the Power Analysis and Sample Size software (2011) before the trial began. However, in view of the small sample size of our study, we defined this trial as a clinical pilot study.

The study protocol was approved by the Institutional Ethics Committee of Nanjing First Hospital (Approval Number: KY20180713-01), and informed consent was obtained from patients' first-degree relatives. The study was also registered at Clinical Trials.gov (ID: NCT03791866). Figure 1 shows the flow diagram of the participants.

Patients

From October 2018 to March 2020, all adult patients (aged 18-70 years) admitted to Department of Critical Care Medicine of Nanjing First Hospital with sepsis diagnosed were enrolled in this study. The diagnostic criteria for sepsis were in compliance with the surviving sepsis guidelines[2,3]. Patients with inflammatory bowel disease, digestive tract hemorrhage, ileus, severe abdominal hypertension (intra-abdominal pressure > 25 mmHg) or abdominal compartment syndrome, malnutrition, immunodeficiency, or chronic organ dysfunction (*e.g.*, hepatic or renal dysfunction), and patients with a history of long-term use of hormones were excluded. All patients received specialized critical care for sepsis as needed, including oxygen administration or mechanical ventilation (MV), antimicrobial therapy, vasopressor administration, fluid resuscitation, glucose control, analgesia and sedation, or renal replacement therapy[1,2,20].

Nutrition protocols

Before EN started, a nasogastric or nasojejunal feeding tube (size 10F, Flocare, Nutricia Ltd) was inserted as needed. The nasojejunal tube was intubated using our novel method of bedside post-pyloric placement[10,21]. The enteral feeding began within 24-48 h of enrollment if there were no contraindications. A peptide-based formula (Peptisorb, Nutricia Ltd) was provided in the first 24-48 h, and if the patients were tolerant, whole protein formula (Nutrison Fibre, Nutricia Ltd) was provided gradually [10]. The goal caloric requirement was determined as 20-25 kcal/kg per day, and the protein need was determined as 1.2-2.0 g/kg/d[9,10,18]. Patients were randomly assigned to receive 30%, 60%, or 100% of goal caloric requirements of nutrition. The EN feeding was started at a slow rate (10-20 mL/h) while carefully monitoring abdominal/ gastrointestinal symptoms[9]. If patients were intolerant because of high gastric residual volume (> 500 mL), diarrhea, nausea, vomiting, or abdominal distension, we slowed down the feeding rate, diluted the feeding concentration, or

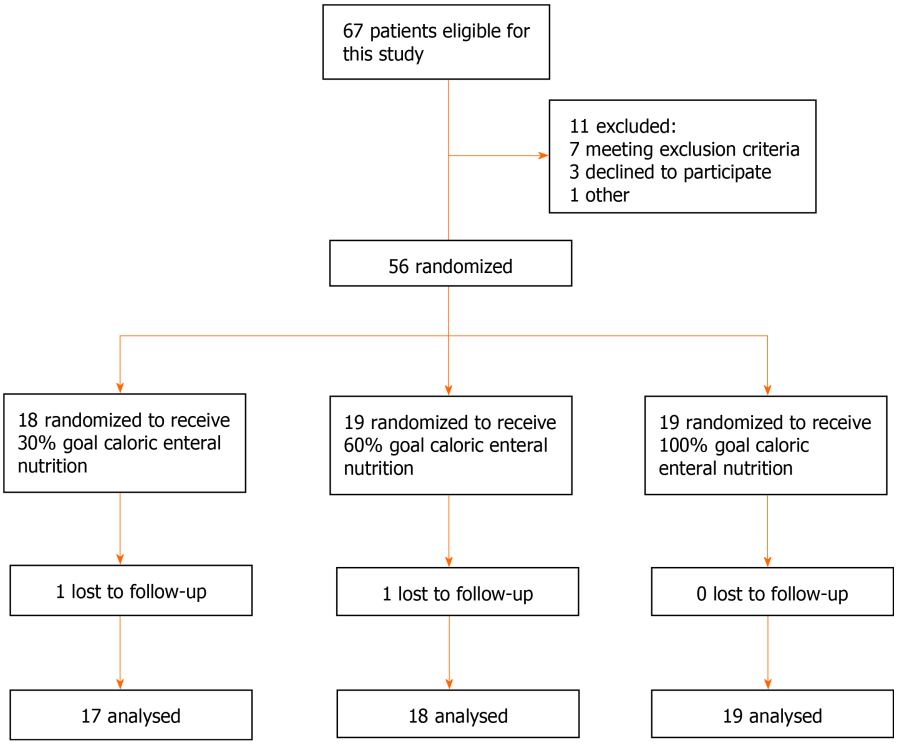


Figure 1 Flow diagram of the participants.

used prokinetic agents.

Parenteral nutrition (PN) was supplemented if the enteral nutrition could not achieve > 60% of the goal caloric requirements after 7 d[8,9,18]. The goal caloric requirement of PN was determined as 20-25 kcal/kg per day, and the calorie/nitrogen ratio was determined as 120-150:1[8,18]. Fifty to seventy percent of the total caloric requirements were supplied by glucose, whereas the provision of lipids was based on serum triglyceride levels. Moreover, sufficient electrolytes, insulin, vitamins, and trace elements were also provided.

Definitions

Sepsis was defined as life-threatening organ dysfunction caused by a dysregulated host response to infection, and septic shock was defined as a subset of sepsis with circulatory and cellular/metabolic dysfunction that was associated with a higher risk of mortality[1-3]. The definition of acute respiratory distress syndrome (ARDS) was based on the Berlin definitions[22,23]. The diagnostic criteria for acute kidney injury (AKI) were in accordance with the 2012 Kidney Disease: Improving Global Outcomes guidelines[24]. Acute gastrointestinal injury (AGI) was defined as a malfunction of the gastrointestinal tract due to acute illness and was categorized into four grades (I to IV) according to its severity[25,26]. AGI grade I was defined as an increased risk of developing gastrointestinal dysfunction or failure (a self-limiting condition); AGI grade II was defined as gastrointestinal dysfunction (a condition that requires interventions); AGI grade III was defined as gastrointestinal failure (gastrointestinal function cannot be restored with interventions); AGI grade IV was defined as marked gastrointestinal failure (a condition that is immediately life-threatening)[25,26].

Feeding intolerance (FI) syndrome was a general term indicating intolerance of enteral feeding for whatever clinical reason (vomiting, high gastric residuals, diarrhea, occurrence or worsening of bowel dilatation, gastrointestinal bleeding, presence of entero-cutaneous fistulas, *etc.*)[25,26]. Diarrhea was defined as having three or more loose or liquid stools per day with a stool weight greater than 200-250 g/d (or greater than 250 mL/d)[26]. High gastric residuals was considered if the gastric residual volume (GRV) was > 500 mL/6 h[26]. Intra-abdominal hypertension (IAH) was defined if intra-abdominal pressure (IAP) was 12 mmHg or higher, confirmed by at least two measurements, 1-6 h apart[26]. Paralysis of the lower GI tract (paralytic ileus) was defined as the inability of the bowel to pass stool due to impaired peristalsis[26]. Clinical signs of paralytic ileus included absence of stool for three or more consecutive days without mechanical obstruction. Multiple organ dysfunction syndrome (MODS)

was defined as the combined dysfunction of two or more organs.

Data collection

On admission to ICU, the baseline clinical data, including age, sex, body mass index, and the etiology of sepsis, were recorded. The acute physiology and chronic health evaluation II (APACHE II) scores and sequential organ failure assessment (SOFA) scores were collected on days 1, 3, and 7 after admission. The AGI grades, number of patients with FI symptoms, frequency of FI symptoms, days to goal calorie, and actual daily caloric intake were also registered. Since there is no "gold standard" to define the malnourished ICU patients[18,27], we used general clinical assessment markers (albumin, prealbumin, IL-6, and IL-10) to reflect nutritional and inflammatory status according to previous reports[27,28]. The levels of albumin, prealbumin, IL-6, and IL-10 in peripheral blood were tested on days 1, 3, and 7 after admission. Meanwhile, the levels of mucosal barrier function biomarkers, including diamine oxidase (DAO), D-lactate, and intestinal fatty acid binding protein (iFABP)[29-31], were also measured. Serum IL-6, IL-10, DAO, D-lactate, and iFABP levels were detected with commercially available Human Quantikine enzyme-linked immunosorbent assay (ELISA) kits (R and D Systems, Bio-Techne Corporation, United States) according to the manufacturer's instructions. The number of patients receiving MV or continuous renal replacement therapy (CRRT) was recorded. The MV-free days, CRRT-free days, and ICU-free days in 28 d of ICU admission, as well as MODS incidence and 28-d mortality, were also collected.

Statistical analysis

The Kolmogorov-Smirnov test was first performed to test the normal distribution of the data. Normally distributed data are expressed as the mean ± SD and were compared by *t* tests. Non-normally distributed data are expressed as the median (interquartile ranges) and were compared by the Mann-Whitney *U* test or the Kruskal-Wallis test. Categorical variables are presented as absolute numbers or percentages and were analyzed using the χ^2 test or Fisher's exact test. To take into account the repeated nature of the variables, analysis of variance (ANOVA) for repeated measurements of the general linear model was implemented. Survival curves for up to 28 d of enrollment were performed using the Kaplan–Meier method and were compared by the log-rank test. IBM Statistical Package for the Social Sciences (SPSS, version 22.0, NY, United States) software was used for statistical analyses, and two-sided $P < 0.05$ was considered statistically significant. The statistical methods of this study were reviewed by Qiao Liu, a biostatistician from the Center for Disease Control and Prevention of Jiangsu Province, China.

RESULTS

As shown in Figure 1, a total of 54 septic patients were enrolled in this study. Seventeen patients were randomly assigned to receive 30% of goal caloric requirements of nutrition (defined as group A), 18 received 60% (defined as group B), and 19 received 100% (defined as group C). The demographic data and clinical parameters of the patients on admission are shown in Table 1. Forty-two (42/54, 77.8%) patients had initial AGI, of whom 13 had AGI grade I, 24 had AGI grade II, and 5 had AGI grade III on admission. Sixteen (16/54, 29.6%) patients had FI symptoms during the first 7 d of enteral feeding. Forty-nine (49/54, 90.7%) patients received MV, and 20 (20/54, 27.0%) patients received CRRT. The 28-d mortality was 25.9% (14/54) in hospital stay.

Gastrointestinal function and nutritional variables

During the 7 d of enteral feeding, no significant differences in the AGI grades were found among the three groups (Figure 2A). The days to goal calorie of group C (2.55 ± 0.82) were significantly longer than those of groups A (3.50 ± 1.51; $P = 0.046$) and B (4.85 ± 1.68; $P < 0.001$) (Figure 2B). However, no difference in the days was found between groups A and B ($P = 0.077$). Figure 2C shows the differences in the actual daily caloric intake among the three groups. The daily caloric intakes of group A were all significantly lower than those of group C during the 7 d of enteral feeding ($P < 0.001$). The daily intakes of group A were significantly lower than those of group B from the 2th day of enteral feeding ($P < 0.01$). The daily intakes of group B were significantly lower than those of group C from the 3th day of enteral feeding ($P < 0.05$).

Table 1 Demographic data and clinical parameters on admission

Variable	Value
Age (yr)	67.0 (63.0-72.3)
Sex (Male:female)	29:25
BMI (kg/m^2)	24.3 (21.5-27.0)
Etiology of sepsis, *n* (%)	
Abdominal infection	27 (50.0)
Thoracic/pulmonary infection	10 (18.5)
Blood stream infection	6 (11.1)
Urinary infection	6 (11.1)
Mucocutaneous infection	3 (5.6)
Other	2 (3.7)
Initial AGI grade, *n* (%)	
I	13 (31.5)
II	24 (55.6)
III	5 (13.0)
APACHEII score	22.0 (19.5-27.8)
SOFA score	9.5 (8.0-11.8)
Feeding intolerance, *n* (%)	16 (29.6)
Need for MV, *n* (%)	49 (90.7)
Need for CRRT, *n* (%)	20 (37.0)
MV-free days	19.5 (1.8-22.8)
CRRT-free days	23.0 (13.3-27.0)
ICU-free days	17.0 (0.0-22.8)
MODS, *n* (%)	20 (37.0)
Death, *n* (%)	14 (25.9)

BMI: Body mass index; AGI: Acute gastrointestinal injury; APACHE II: Acute physiology and chronic health evaluation II; SOFA: Sequential organ failure assessment; MV: Mechanical ventilation; CRRT: Continuous renal replacement therapy; ICU: Intensive care unit; MODS: Multiple organ dysfunction syndrome.

As shown in Table 2, 16 (16/54, 29.6%) patients had FI symptoms during the first 7 d of enteral feeding. The proportion of patients with FI symptoms of group A was significantly lower than that of group C (11.8% *vs* 52.6%, $P = 0.019$). However, no difference in the proportion was found between groups B and A ($P = 0.658$) or C ($P = 0.057$). Table 3 shows the differences in the incidence of single FI symptom among the three groups. A total of 39 FI symptoms were observed during the 7 d of enteral feeding, and diarrhea was the most common manifestation. Although the total frequency of FI symptoms was different ($P = 0.009$) among the three groups, no difference in the incidence of single symptom (except for diarrhea, $P = 0.046$) was found. Moreover, there was no difference in the incidence of all symptoms between groups A and B.

Comparison of the levels of intestinal barrier biomarkers among the three groups is presented in Figure 3. The serum concentrations of DAO, D-lactate, and iFABP of group B were significantly lower than those of group A ($P < 0.05$) on the 7th day of feeding. The serum concentrations of the three biomarkers of group C were only numerically lower than those of group A on the 7th day of feeding ($P > 0.05$). Figure 4 shows the differences in the levels of nutritional and inflammatory markers among the three groups. The prealbumin level (mg/L) of group A was lower than those of groups B (71.09 ± 20.23 *vs* 103.33 ± 40.45, $P = 0.031$) and C (71.09 ± 20.23 *vs* 110.62 ± 37.05, $P = 0.008$) on the 7th day of feeding. The IL-6 level (pg/L) of group A was higher than that

Table 2 Number of patients with feeding intolerance and clinical severity and outcome variables

	Group A (*n* = 17)	Group B (*n* = 18)	Group C (*n* = 19)	*P* value
Feeding intolerance, *n* (%)	2 (11.8)	4 (22.2)	10 (52.6)	0.019
MV-free days	19.0 (0.0-21.0)	22.0 (1.0-25.0)	18.0 (4.0-22.5)	0.347
CRRT-free days	20.0 (12.0-28.0)	26.0 (7.8-28.0)	22.0 (14.5-28.0)	0.778
ICU-free days	19.0 (0.0-23.0)	19.0 (0.0-21.8)	16.0 (0.0-21.0)	0.572
MODS, *n* (%)	6 (35.3)	8 (44.4)	6 (31.6)	0.709
Death, *n* (%)	4 (23.5)	5 (27.8)	5 (26.3)	0.856

Group A: Patients were assigned to receive 30% of goal caloric requirements of enteral nutrition; Group B: Patients were assigned to receive 60% of goal caloric requirements of enteral nutrition; Group C: Patients were assigned to receive 100% of goal caloric requirements of enteral nutrition. MV: Mechanical ventilation; CRRT: Continuous renal replacement therapy; ICU: Intensive care unit; MODS: Multiple organ dysfunction syndrome.

Table 3 Incidence of feeding intolerance symptoms, *n* (%)

	Group A (*n* = 17 × 7)	Group B (*n* = 18 × 7)	Group C (*n* = 19 × 7)	*P* value
Feeding intolerance	6 (5.0)	11 (8.7)	22 (16.5)	0.009
Nausea or vomiting	2 (1.7)	3 (2.4)	3 (2.3)	0.758
Diarrhea	2 (1.7)	2 (1.6)	8 (6.0)	0.046
Abdominal pain	0 (0.0)	0 (0.0)	1 (0.8)	0.238
Abdominal distention	1 (0.8)	2 (1.6)	6 (4.5)	0.054
High gastric residuals	1 (0.8)	2 (1.6)	2 (1.5)	0.653
IAH	0 (0.0)	1 (0.8)	1 (0.8)	0.422
Paralytic ileus	0 (0.0)	1 (0.8)	1 (0.8)	0.444

Group A: Patients were assigned to receive 30% of goal caloric requirements of enteral nutrition; Group B: Patients were assigned to receive 60% of goal caloric requirements of enteral nutrition; Group C: Patients were assigned to receive 100% of goal caloric requirements of enteral nutrition. IAH: Intra-abdominal hypertension.

of group B (115.54 ± 72.37 *vs* 62.00 ± 35.59, $P = 0.028$) on the 7th day of feeding. However, no difference in the IL-6 level was found between groups B and C ($P = 0.126$). No significant differences in the albumin and IL-10 levels were found among the three groups.

These results indicated that 60% of goal caloric requirements of enteral feeding may reduce the injury of intestinal barrier and improve the nutritional and inflammatory status without increasing the incidence of FI symptoms compared with 30% of goal caloric requirements. On the other hand, 60% of goal caloric requirements may decrease the incidence of FI symptoms without deteriorating the intestinal barrier function and nutritional status compared with 100% of goal caloric requirements.

Clinical severity and outcome variables

During the 7 d of ICU admission, no significant differences in the APACHE II scores or SOFA scores were found among the three groups ($P > 0.05$; Figure 5A and B). As shown in Table 2, no differences in the MV-free days, CRRT-free days, ICU-free days, MODS incidence, or 28-d mortality were found among the three groups ($P > 0.05$). The results of survival analysis (Figure 5C) also confirmed that the survival probability of patients was not affected by different goal caloric requirements (30%, 60%, and 100%) of enteral feeding in the acute phase of sepsis.

DISCUSSION

This clinical pilot study investigated the effects of different goal caloric requirements (30%, 60%, and 100%) of enteral feeding on gastrointestinal function and outcome of

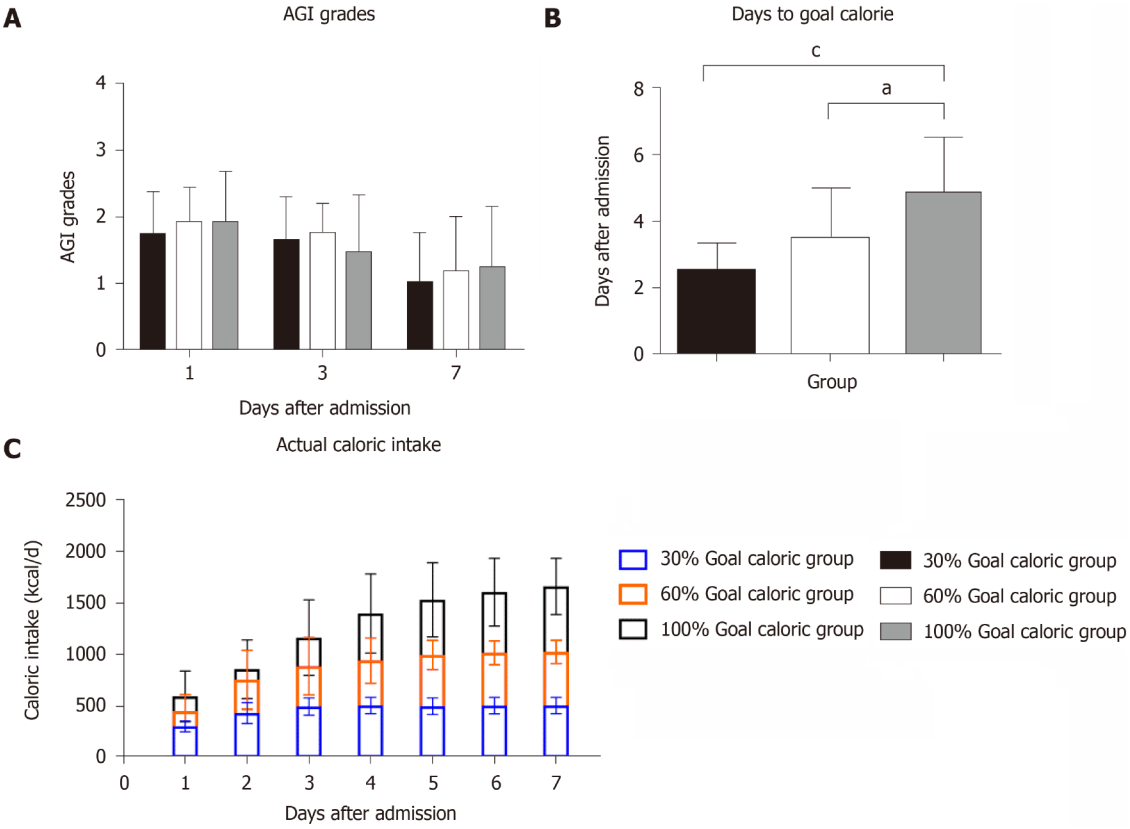


Figure 2 Comparisons of acute gastrointestinal injury grades (A), days to goal calorie (B), and actual daily caloric intake (C) among the three groups. $^{a}P < 0.05$, $^{c}P < 0.001$. AGI: Acute gastrointestinal injury.

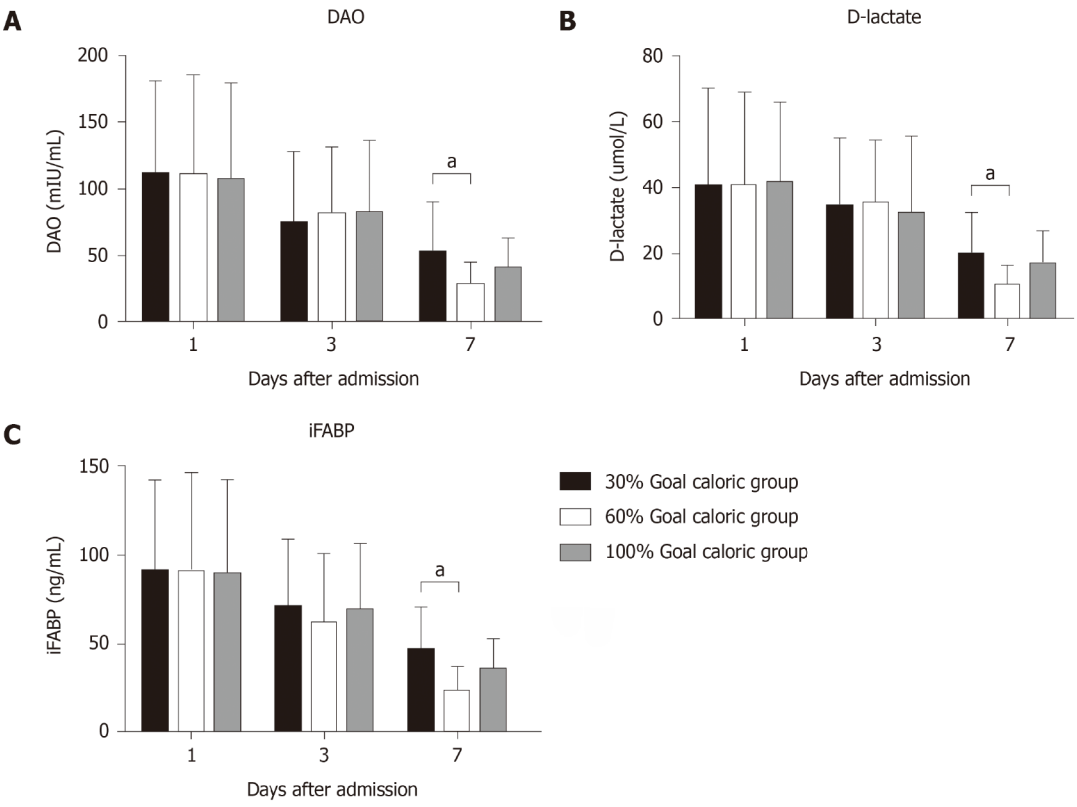


Figure 3 Comparisons of serum levels of diamine oxidase (A), D-lactate (B), and intestinal fatty acid binding protein (C) among the three groups. $^{a}P < 0.05$. DAO: Diamine oxidase; iFABP: Intestinal fatty acid binding protein.

sepsis in its acute phase. We found that 60% of total enteral feeding may improve the

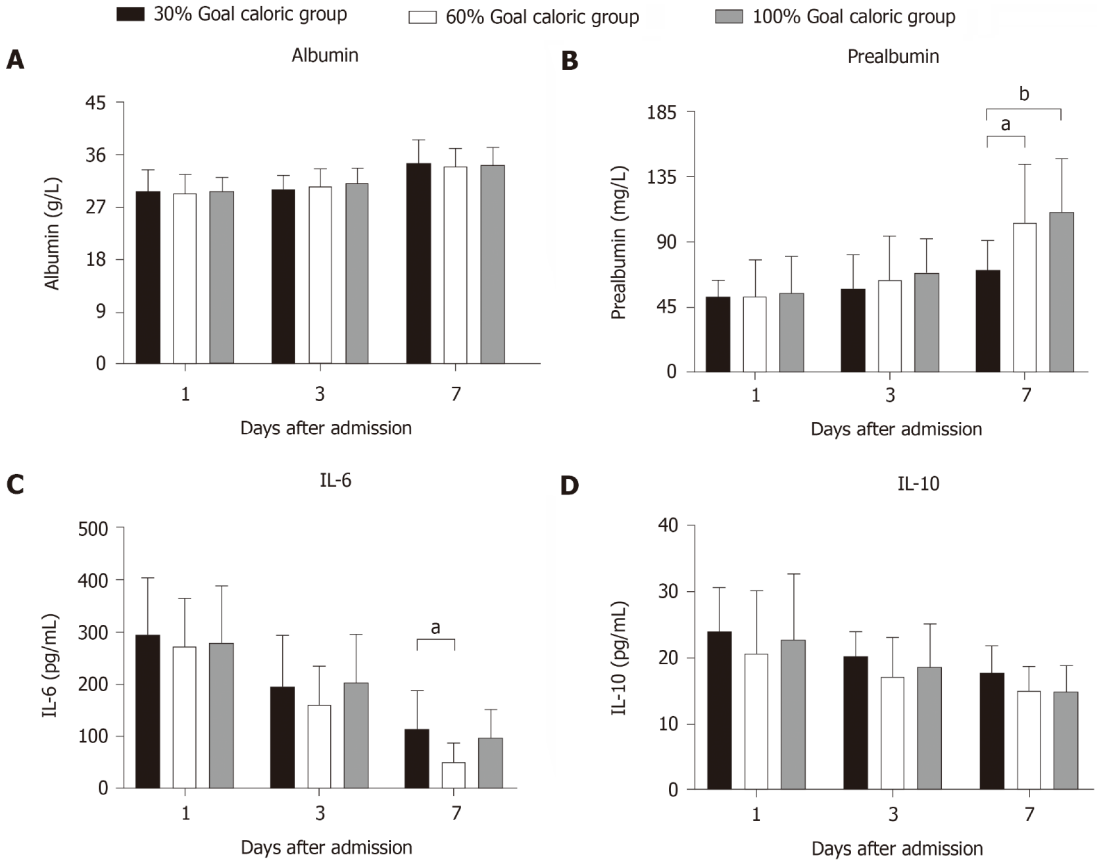


Figure 4 Comparisons of serum levels of albumin (A), prealbumin (B), IL-6 (C), and IL-10 (D) among the three groups. $^{a}P < 0.05$, $^{b}P < 0.01$.

Nutrition therapy is a crucial medical treatment during the acute phase of critical illness. Unfortunately, recent trial findings and guideline recommendations continued to be conflicting, making the translation of evidence into practice challenging[6,27]. No complete consensus has been reached on the estimation of energy expenditure, timing of feeding, choice of enteral or parenteral nutrition, protein and energy requirement, and monitoring of nutrition in sepsis. A literature review suggested that full feeding in the acute phase of critical illness did not provide an advantage over trophic feeding and may be harmful[6]. In sepsis, more or less nutrition delivery is an updated focus under exploration. Van Niekerket al[32] suggested that less nutrition supply may be beneficial during sepsis. However, the PROCASEPT study indicated that low protein intake or caloric restriction may not be of benefit in septic patients[33,34], and overfeeding during days 4-7 was related to a lower 6-mo mortality, compared with low caloric intake[34]. Due to the lack of direct or indirect calorimetric system of energy metabolism, we used the most commonly predictive equations to estimate the goal energy requirement of patients (20-25 kcal/kg per day) according to ASPEN and ESPEN guidelines[8,18]. We found that more (100%) or less (30% or 60%) enteral nutrition was not associated with the clinical outcomes, including 28-d mortality. This finding was not contradictory to the previous reports[13,17]. Nevertheless, moderate (60%) enteral nutrition may be beneficial to the gastrointestinal function and feeding tolerance during acute phase of sepsis.

Gastrointestinal dysfunction is common and closely related to adverse outcomes in critically ill patients[25]. AGI is often caused by severe trauma, infection, sepsis, shock, and other critical diseases[25]. Accompanied by that, intestinal epithelial cell damaged, mucosal permeability increased, intestinal flora translocated, and then intestinal infection and MODS developed[25,26]. Therefore, improving the intestinal barrier injury was considered to be an important measure in the treatment of sepsis. EEN was proven to regulate excessive immune response and maintain the intestinal barrier function of critically ill patients[6,9,27]. The results of our study were consistent with the previous studies. However, we found that full enteral feeding increased the incidence of FI symptoms without improving the intestinal barrier function and nutritional and inflammatory status, compared with underfeeding. This phenomenon also revealed that enteral underfeeding, especially 60% of goal caloric requirements,

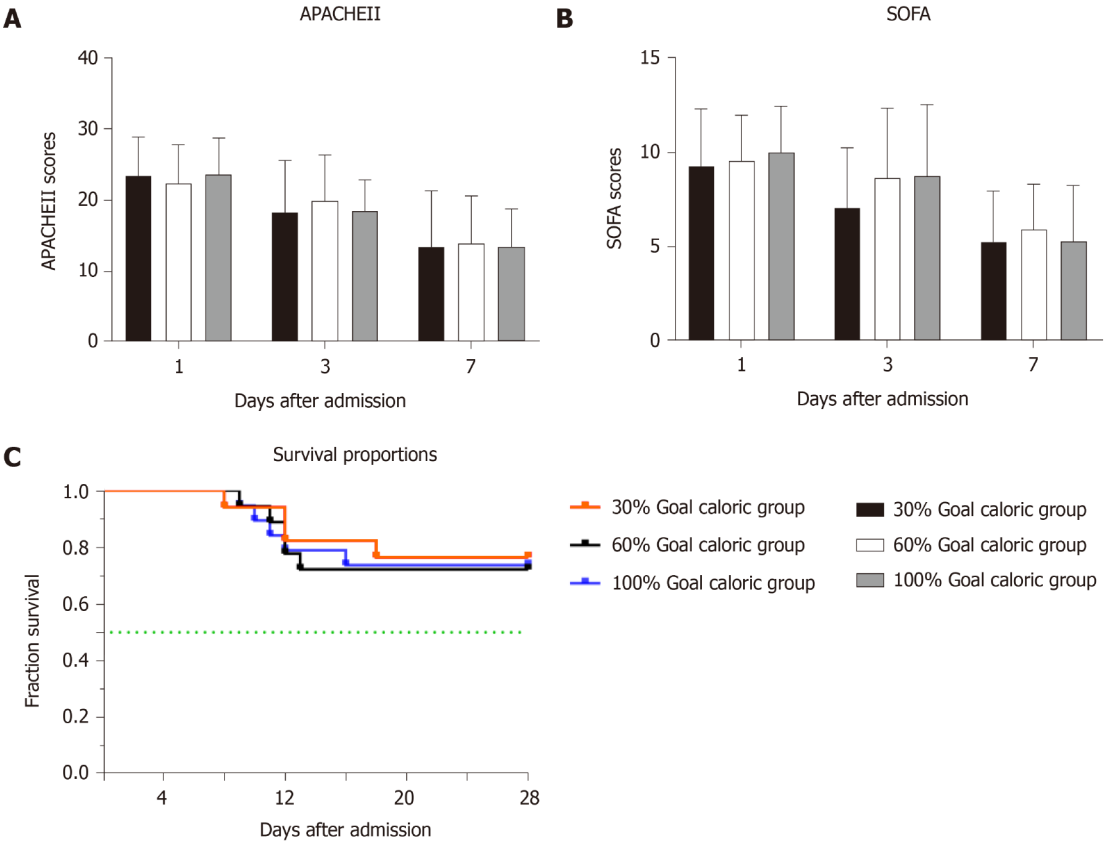


Figure 5 No significant differences in the acute physiology and chronic health evaluation II scores (A), sequential organ failure assessment scores (B), or survival probability within 28 d (C) were found among the three groups. APACHEII: Acute physiology and chronic health evaluation II; SOFA: Sequential organ failure assessment.

may be beneficial to sepsis. The underlying mechanisms of permissive underfeeding were proposed by a recent review[32]: Suppression of early feeding may result in a synergistic potentiation of catabolism and then promote cell survival and enhance immune function in sepsis. But further clinical and animal studies are required to verify this theory.

FI is the most commonly encountered challenge during enteral feeding in critically ill patients. Hu *et al*[35] reported that FI incidence was approximately 24% during the first week of ICU stay, and persistent FI was an independent risk factor for mortality in critically ill patients. A newly large-scale analysis of a multicenter, multiyear database showed that burn, gastrointestinal dysfunction, and sepsis were more likely to result in enteral feed intolerance, compared with respiratory-related illness, and enteral feed intolerance is associated with lower enteral nutrition delivery and worse clinical outcomes in mechanically ventilated critically ill patients[36]. Hence, prevention and optimal management of FI may improve nutrition delivery and clinical outcomes in important "at risk" populations. The FI incidence of this study was 29.6% during the first 7 d of enteral feeding in sepsis. Compared with full enteral feeding, 60% or 30% of total enteral feeding decreased the incidence of FI symptoms. However, 30% of total enteral feeding did not improve the nutritional and inflammatory status compared with 100% or 60% of total enteral feeding. It means that moderate underfeeding (60% of goal requirement) might be a preferable prevention and management strategy for FI and undersupply in sepsis. No difference in 28-d mortality among the three groups of different enteral intakes might be explained by our small sample size and single disease design, and large-scale prospective clinical studies are needed.

Some limitations of this study should be discussed. Due to our single-center design and small sample size, the results may not be generalizable, and the conclusions should be confirmed by large-scale clinical prospective trials. Moreover, the effects of different protein intake on gastrointestinal function and outcomes were not investigated. Finally, because our variables were only recorded for 1 wk, the later effects of enteral feeding on gastrointestinal function and outcomes should be researched in future clinical trials.

CONCLUSION

This clinical pilot study found that early moderate underfeeding (60% of goal requirements) could improve the intestinal barrier function and nutritional and inflammatory status without increasing the incidence of FI symptoms in sepsis. No difference in the clinical outcomes was found among the three groups of different enteral intakes. However, further large-scale prospective clinical trials and animal studies are required to test our findings. Moreover, the effects of different protein intake on gastrointestinal function and outcomes should also be investigated in future work.

ARTICLE HIGHLIGHTS

Research background

Few studies have investigated the effects of enteral underfeeding on gastrointestinal function in the acute phase of sepsis.

Research motivation

No consensus on goal enteral caloric intake has been reached in sepsis.

Research objectives

To investigate the effects of different goal caloric requirements of enteral nutrition on the gastrointestinal and outcomes in the acute phase of sepsis.

Research methods

Patients were randomly assigned to receive 30%, 60%, or 100% of goal caloric requirements of enteral nutrition in this prospective pilot clinical trial. The gastrointestinal function, nutritional and inflammatory markers, clinical severity, and outcome variables were recorded.

Research results

Early moderate enteral underfeeding (60% of goal requirements) could improve the intestinal barrier function and nutritional and inflammatory status without increasing the incidence of feeding intolerance symptoms in sepsis. No significant differences in the clinical outcome variables or 28-d mortality were found.

Research conclusions

Early moderate enteral underfeeding could improve the intestinal barrier function and nutritional and inflammatory status without increasing the incidence of feeding intolerance symptoms in sepsis.

Research perspectives

It is necessary to explore an optimal goal of enteral feeding to improve the acute intestinal injury in sepsis. In this study, we investigated the effects of different goal caloric requirements (30%, 60%, and 100%) of enteral nutrition on the gastrointestinal function in sepsis. Further large-scale prospective clinical trials and animal studies are required to test our findings.

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ORIGINAL ARTICLE

Observational Study

Advanced glycation end product: A potential biomarker for risk stratification of non-alcoholic fatty liver disease in ELSA-Brasil study

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Abstract

BACKGROUND

Liver diseases are associated with the excess formation of advanced glycation end products (AGEs), which induce tissue inflammation and oxidative damage. However, the trend of oxidative marker levels according to the steatosis grade in non-alcoholic fatty liver disease (NAFLD) is unclear.

AIM

To compare serum AGE levels between participants with NAFLD accordingly to steatosis severity in the baseline ELSA-Brasil population.

METHODS

In 305 individuals at baseline ELSA-Brasil, NAFLD-associated steatosis was classified by ultrasound hepatic attenuation. The participants were grouped according to the severity of steatosis: mild and moderate/severe pooled. The measurement of serum fluorescent AGE concentrations was based on spectrofluorimetric detection. Serum AGE content and clinical and laboratory characteristics of the participants were compared between groups. The correlation between

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serum AGE levels and the grade of steatosis was analyzed. Logistic regression analysis was used to investigate the relationship between serum AGE levels and steatosis severity. A P value < 0.05 was considered statistically significant.

RESULTS

According to the steatosis severity spectrum in NAFLD, from mild to moderate/severe, individuals with the most severe steatosis grade had a higher incidence of metabolic syndrome (63% *vs* 34%, $P \leq 0.001$), diabetes mellitus (37% *vs* 14%, $P \leq 0.001$), and high cholesterol levels (51% *vs* 33%, $P < 0.001$). Moreover, individuals with increasing severity of steatosis presented increasing waist circumference, body mass index, systolic and diastolic blood pressure, fasting blood glucose, glycated hemoglobin, insulin, triglycerides, alanine aminotransferase, gamma-glutamyl transferase, C-reactive protein, and uric acid levels and lower high-density lipoprotein. Higher serum AGE content was present in the moderate/severe group of individuals than in the mild group ($P = 0.008$). In addition, the serum AGE levels were correlated with the steatosis grade in the overall sample (rho = 0.146, $P = 0.010$). Logistic regression analysis, after adjusting for confounding variables, showed that subjects with higher serum AGE content had a 4.6-fold increased chance of having moderate or severe steatosis when compared to low levels of serum AGEs. According to the results of the receiver operator characteristic curves analyses (areas under the curve, AUC = 0.83), AGEs could be a good marker of steatosis severity in patients with NAFLD and might be a potential biomarker in predicting NAFLD progression, strengthening the involvement of AGE in NAFLD pathogenesis.

CONCLUSION

NAFLD-associated steatosis was associated with serum AGE levels; therefore, plasmatic fluorescent AGE quantification by spectroscopy could be a promising alternative method to monitor progression from mild to severe NAFLD accordingly to steatosis grade.

Key Words: Advanced glycation end products; Non-alcoholic fatty liver disease; Steatosis; ELSA-Brasil study; Spectroscopy

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Core Tip: We evaluated the relationship between fluorescent advanced glycation end product (AGE) levels and the severity of non-alcoholic fatty liver disease (NAFLD)-associated steatosis. We evaluated 305 subjects with NAFLD from Rio de Janeiro in the baseline ELSA-Brasil population grouped according to the steatosis stratification: mild and moderate/severe pooled. Serum AGE levels were correlated with the steatosis grade in the overall sample. The severity of NAFLD-associated steatosis was associated with serum AGE levels; therefore, plasmatic fluorescent AGE quantification by spectroscopy could be a promising alternative method for classifying and grading NAFLD accordingly to hepatic steatosis.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is characterized by abnormal liver fat accumulation in the absence of significant alcohol consumption or secondary causes of fat accumulation[1,2]. NAFLD includes a large spectrum of clinical phenotypes, from

quality classification
Grade A (Excellent): 0
Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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non-alcoholic fatty liver to non-alcoholic steatohepatitis (NASH), in which inflammation and hepatocyte injury are present. Individuals with NAFLD can develop progressive fibrosis, liver cirrhosis, hepatocellular carcinoma, and end-stage liver disease[3]. In Western populations, NAFLD is rapidly becoming the top reason for liver transplants[4,5].

The prevalence of NAFLD worldwide is estimated to be 25%, with the highest prevalence in South America (30.45%). This condition is considered the leading cause of chronic liver disease in the United States and Europe[6,7]. Currently, there is a strong bidirectional association between NAFLD and metabolic syndrome (MetS) when components of MetS, such as insulin resistance and hypertriglyceridemia, increase an individual's chance of developing NAFLD[8-10]. Furthermore, metabolic disorders such as diabetes mellitus, dyslipidemia, and obesity have also been associated with NAFLD[1,11,12].

The most common imaging techniques used to diagnose NAFLD are ultrasonography, magnetic resonance, computerized tomography, and controlled attenuation parameter[13,14]. NAFLD can be histologically distinguished from alcoholic steatohepatitis based on histological markers in liver biopsy: Lobular inflammation, hepatocyte ballooning, portal granulocytic inflammation, and Mallory-Denk bodies [15]. Clinical settings and population studies with NAFLD commonly use conventional B-mode ultrasonography because it is non-invasive, easy to use, inexpensive, and widely available[16,17]. Despite the many methods available to assess NAFLD, the pathophysiology of NAFLD is extremely complex and has only been partially elucidated. Although laboratory tests, imaging techniques, and histology play a pivotal role in NAFLD diagnosis, novel alternative methods to diagnose and monitor NAFLD progression are critically needed.

According to the multiple-hit hypothesis, several factors in parallel act synergistically, causing metabolic and molecular alterations that lead to the development and progression of NAFLD[18]. Accumulation of fat in the liver is considered one of the first hits; however, the progression of the disease requires the involvement of other secondary factors, such as insulin resistance, inflammatory mediators, gut microbiome, mitochondrial dysfunction, genetics, nutritional and environmental factors, and/or ER stress[18-20]. Among the "multiple hits", advanced glycation end products (AGEs) have been observed by several studies to play a critical role in the pathogenesis of NAFLD[19,21,22].

AGEs are products of non-enzymatic reactions of reducing sugars or oxidized lipids with amino groups in proteins, lipids, and nucleic acids[23]. In addition to being produced in an endogenous manner, AGEs are also present in foods highly processed or dry heated at high temperatures[24]. Increased AGE levels can trigger loss of normal structure and protein dysfunction due to abnormal cross-linking between intracellular and extracellular proteins. Moreover, the activation of the receptor for AGE (RAGE) by binding AGEs can trigger an increase in inflammation and oxidative stress[25] and participate in insulin resistance, liver injury, and fibrosis[22,26]. AGEs are increased in several pathological conditions such as Alzheimer's disease[27], cardiovascular diseases[28-30] diabetes mellitus[31-33], and liver diseases[34]. We have previously shown that activation of the AGE-RAGE axis, oxidative stress, and inflammation could have a role in microcirculatory alterations in NAFLD[35]. The increased accumulation of AGEs in the serum or liver tissue of NASH patients could induce cell damage and necrotic-type hepatocyte death[36]. AGEs can leak outside of cells and induce inflammatory or fibrotic responses in adjacent cells[36]. To date, there is a lack of clinical studies investigating the role of AGEs in the pathogenesis of NAFLD. Given this, it is of great interest to address the specific pathophysiological mechanisms underlying NAFLD-associated steatosis association with AGEs levels in a large and mixed population cohort. Therefore, in this study, we addressed whether steatosis severity was associated with serum AGE levels in the baseline data from the Brazilian Longitudinal Study of Adult Health (ELSA-Brasil) study. We propose plasmatic fluorescent AGE quantification by spectroscopy as a promising alternative method to monitor progression of NAFLD accordingly to steatosis severity.

MATERIALS AND METHODS

Study population

This is a cross-sectional study addressing the association between NAFLD and AGEs in the baseline ELSA-Brasil population; a cohort of active or retired employees of both sexes, aged between 35 and 74 years, totaling 15105 civil servants living in six cities in

Brazil (Belo Horizonte, Porto Alegre, Rio de Janeiro, Salvador, São Paulo, and Vitoria) from predefined universities or research institutes[37]. In the present study, subjects from Rio de Janeiro were included (n = 305) (Figure 1). Exclusion criteria were as follows: no information on serum AGE content, no information on alcohol consumption, or excessive alcohol consumption (> 210 g/wk for men or 140 g/wk for women). In addition, subjects who had no NAFLD evaluation, poor ultrasound image quality, or normal hepatic deep-beam attenuation (complete viewing) were excluded. This study was approved by all the institutional review boards of the participating institutions, which conforms to the ethical guidelines of the 1975 Declaration of Helsink, and all participants provided informed consent.

Data collection

Clinical and anthropometric examination data were collected on-site by questionnaires and clinical exams between August 2008 and December 2010[38]. The questionnaire included information on age at the baseline visit (years), gender, alcohol consumption (never used, ex-user or user), and smoking habit (non-smoker or smoker). Waist circumference (cm), weight (kg), and height (cm) were measured according to standard techniques[39]. Body mass index (BMI) was calculated by dividing body weight by squared height in meters (kg/m^2). Blood pressure was measured using a validated Omron HEM 705CPINT oscillometer device (Omron Co, Kyoto, Japan) three times with a 1 min interval in the sitting position and the mean of the last two measurements defined the blood pressure value.

Participants' sera were collected after 12 h of fasting, centrifuged, and stored at -70 °C until analysis. Gamma-glutamyl transferase (GGT) was measured using a kinetic colorimetric assay; aspartate aminotransferase (AST) and alanine aminotransferase (ALT) by enzymatic assay (ADVIA Chemistry, Siemens, Deerfield, United States); uric acid by colorimetric enzyme (uricase) method; total cholesterol, high-density cholesterol, and triglycerides by the enzymatic colorimetric method. Low-density cholesterol was calculated by the Friedewald equation if triglycerides ≤ 400 mg/dL or directly measured by enzymatic colorimetric method otherwise; glucose was measured by hexokinase method (ADVIA Chemistry, Siemens, Deerfield, United States), glycated hemoglobin (HbA1c) by high-pressure liquid chromatography (Bio-Rad Laboratories, Hercules, CA, United States), fasting insulin was determined by immunoenzymatic assay (ELISA); and high sensitivity C reactive protein by immunochemistry nephelometry (BN II Siemens® nephelometer). Microalbuminuria was measured *via* an immunochemical assay (nephelometry) (Dade Behring). Creatinine levels were measured using an enzymatic colorimetric assay (ADVIA 1200, Siemens, Deerfield, United States). Diabetes was defined by previous medical history of diabetes, antidiabetic drug use, 2 h plasma glucose ≥ 200 mg/dL, fasting plasma glucose ≥ 126 mg/dL, or glycated hemoglobin ≥ 6.5%. High cholesterol was defined as the use of medication to treat dyslipidemia or low-density lipoprotein cholesterol ≥ 130 mg/dL. The presence of MetS was defined according to the NCEP ATP III definition, which requires three or more of the following five criteria: waist circumference ≥ 88 cm for women and ≥ 102 cm for men; antihypertensive drug use or systolic blood pressure ≥ 130 mmHg or diastolic ≥ 85 mmHg; triglycerides ≥ 150 mg/dL; high-density lipoprotein (HDL) cholesterol < 50 mg for women and < 40 mg for men; and fasting glucose ≥ 100 mg/dL with oral or insulin treatment using hypoglycemic agents.

Plasma AGEs

Measurement of fluorescent AGE concentrations was based on spectrofluorimetric detection[40]. Serum was diluted 200-fold with phosphate-buffered saline (KH_2PO_4 1.06 mmol/L, NaCl 155.10 mmol/L, and $Na_2HPO_4 \cdot 7H_2O$ 2.97 mmol/L, pH 7.4) and homogenized using a vortex mixer for 10 s, measured at an emission wavelength of 445 nm and at an excitation wavelength of 370 nm (SpectraMax M5 ELISA Microplate Reader, Molecular Devices, Acton, MA, United States). A solution of BSA (1 mg/mL in 0.1 N NaOH) was used as a reference and its fluorescence intensity was defined as one unit of fluorescence. The amount of fluorescence of the patient serum sample was measured at a protein concentration of 1 mg/mL and expressed in arbitrary units after normalization with the native BSA preparation[41].

Hepatic imaging and NAFLD classification

Ultrasound has been recommended as the first-line method for evaluating hepatic steatosis due to its safety, low cost, and noninvasiveness[42,43]. Among the most common ultrasonography parameters for assessing NAFLD, we have chosen hepatic attenuation of the ultrasound beam because of its better diagnostic performance as

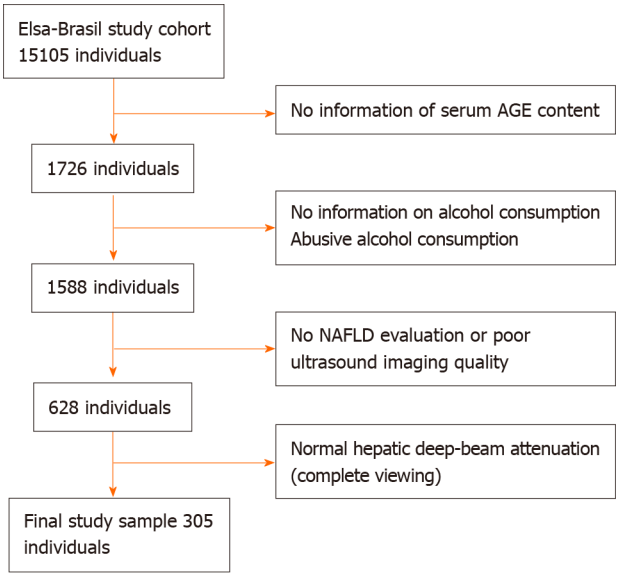


Figure 1 Flow chart of the selection of the eligible study population in the final analysis. AGE: Advanced glycation end product; NAFLD: Non-alcoholic fatty liver disease.

previously described[44]. A standard B-mode ultrasound evaluation was performed using a visual grading system based on the degree of loss of definition of the diaphragm posterior to the right hepatic lobe. Liver ultrasound examinations were performed by previously trained operators using a high-resolution B-mode scanner (SSA-790A, Aplio XG, Toshiba Medical System, Tokyo, Japan) and a convex array transducer (model PVT-375BT), with a central frequency of–3.5 MHz and a fundamental frequency of 1.9-5.0 MHz, the same models of equipment used by the participants at enrollment. After the acquisition process, the B-mode hepatic ultrasound images were read, and the quality control protocol was verified by a senior ultrasound radiologist following a standardized protocol[44]. The presence of steatosis was classified as mild (partial, *i.e.*, > 50% visualization of the diaphragm), moderate (partial, *i.e.*, < 50% visualization of the diaphragm), or severe (no visualization of the diaphragm), as previously validated[44].

Serum biomarkers

Liver steatosis predictive models previously reported in the literature were used to compare the effect of NAFLD classification method on the associations based on fatty liver index (FLI) and hepatic steatosis index (HSI), an algorithm for the prediction of fatty liver in the general population. The FLI is a prevalent biomarker panel consisting of BMI, waist circumference, triglycerides, and gamma-glutamyl transferase for identifying NAFLD, with a total score varying between 0 and 100. The presence of liver steatosis was defined as an FLI ≥ 60, and the absence was defined as FLI < 30[45]. The FLI was calculated according to the following algorithm: FLI = ey/(1 + ey) × 100, where y = 0.953 × ln (triglycerides, mg/dL) + 0.139 × BMI, kg/m^2 + 0.718 × ln (GGT, U/L) + 0.053 × waist circumference, cm – 15.745.

The HSI is a biomarker panel consisting of BMI, diabetes, and the ALT/AST ratio. Liver steatosis was defined as HSI ≥ 36[46]. HSI = 8 × ALT/AST ratio + BMI (+ 2, if diabetes mellitus; + 2, if female).

The atherogenic index (AI), a biomarker used to predict the susceptibility of individuals for developing cardiovascular diseases and atherosclerosis, is strongly associated with NAFLD, which can be used in the auxiliary diagnosis of NAFLD. AI consists of the logarithm of the molar ratio of triglyceride to HDL cholesterol. According to previous studies, AI was stratified into three groups: low (< 0.11), intermediate (0.11-0.21), and high (> 0.21) risk[47].

Statistical analysis

The participants were grouped according to the severity of NAFLD-associated steatosis: mild, and moderate/severe pooled. Clinical and laboratory characteristics were described by median (interquartile range) for continuous variables and by frequency (percentage) for categorical variables. The serum AGE content of the participants was compared across the steatosis stratification. Given the ordinal nature

of steatosis stratification groups, we ran trend analysis using Jonckheere-Terpstra for continuous variables and Cox-Mantel-Haenszel for categorical variables. The Spearman correlation coefficient was used to analyze correlations between serum AGE levels and the grade of steatosis. A P value < 0.05 was considered statistically significant. A logistic regression analysis was used to investigate the relationship between hepatic steatosis severity (moderate/severe pooled) and high levels of serum AGEs (AGEs ≥ 0.85, according to the median of AGE values in the study population). Odds ratios (ORs) were obtained from logistic regression analysis, and the results were presented as ORs with a 95% confidence interval (CI). NAFLD-associated steatosis severity was the dependent variable. In the first model, AGEs were the independent variables; in the second model, the first model plus gender; in the third model, the second model plus gamma-glutamyl transferase levels; in the fourth model, the third model plus altered fasting blood glucose (110 ≤ 125 mg/dL or ≥ 126 mg/dL); in the fifth model, the fourth model plus high cholesterol. This study achieved this model's performance by three folder cross-validations, including sensitivity, specificity, and accuracy. The receiver operator characteristic curves (ROCs) were drawn, which can assess the logistic regression model's prediction performance. Data analysis was performed using R version 3.6.2 (R Project for Statistical Computing) and run in RStudio version 1.2.5033 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Of the 305 individuals from the overall sample, 56% were male, and the median age was 51 (45-55) years. The median (interquartile) serum AGE content was 0.85 (0.76-0.96). Mild steatosis was present in 182 (60%), moderate in 111 (36%), and severe in 12 (4%) individuals. The AGE levels based on NAFLD-associated steatosis stratification were 0.82 (0.75-0.92), 0.86 (0.79-0.98), and 0.98 (0.82-1.10) for mild, moderate, and severe steatosis, respectively. For the subsequent analyses, due to the low frequency of severe steatosis, the moderate and severe individuals were pooled into a single group with 123 (40%) individuals.

Table 1 displays the characteristics of the participants. According to NAFLD-associated steatosis grade, from mild to moderate/severe , individuals were older, but no difference was found between genders. No difference was observed in smoking habits or use of alcohol. According to the NCEP ATP III definition, individuals with the most severe forms of steatosis had a higher prevalence of MetS (63% *vs* 34%, $P \leq 0.001$), diabetes mellitus (37% *vs* 14%, $P \leq 0.001$), and high cholesterol (51% *vs* 33%, $P \leq 0.001$). Similarly, individuals with increasing severity of steatosis presented increasing waist circumference, BMI, systolic and diastolic blood pressure, fasting blood glucose, glycated hemoglobin, insulin, triglycerides, ALT, GGT, C-reactive protein, and uric acid levels and lower HDL. There were no significant differences in total cholesterol, AST, microalbuminuria, creatinine, sodium, and potassium levels among groups (Table 1).

Table 2 shows the association between the FLI, HIS, and AI with the severity of NAFLD-associated steatosis. Notably, increased degrees of steatosis resulted in increased values of the evaluated indexes (Table 2).

Higher serum AGE content was present in the moderate/severe group of individuals than in the mild steatosis group ($P = 0.008$) (Figure 2). In addition, the serum AGE levels were correlated with the steatosis grade in the overall sample (rho = 0.146, $P = 0.010$).

Logistic regression analysis was performed to verify if subjects with higher serum AGE content were more likely to have severe forms of NAFLD accordingly to steatosis levels (Table 3). The results showed that only high AGE serum levels (OR: 2.65, 95%CI: 1.05-6.92) was an independent risk factor for severe NAFLD-associated steatosis, and all four models showed a consistent relationship with the severity of steatosis in NAFLD (Table 3). The risk of moderate or severe NAFLD-associated steatosis in the highest serum AGE content was 239% (OR: 3.39; 95%CI: 1.24-9.98) after adjustment for sex. A similar association was also observed in model 2 after additional adjustment for GGT levels. Model 3 was also associated with an increased risk of severe forms of NAFLD-associated steatosis after additional adjustment for hyperglycemia (OR: 4.53, 95%CI: 1.44-16.28). In model 4, the risk was 4.6-fold higher after adjusting for hypercholesterolemia (OR: 4.67, 95%CI: 1.46-17.17).

To further confirm the predictive power of high AGE levels for NAFLD-stratification accordingly to hepatic steatosis , ROC analysis for the diagnostic value for steatosis was done (Figure 3). The areas under the curve (AUC) for AGEs in model 4 of

Table 1 Clinical characteristics according to non-alcoholic fatty liver disease-associated steatosis severity

	Mild (*n* = 182)	Moderate/severe (*n* = 123)	*P* value[1]
Age (yr)	50 (44-54)	52 (45-57)	0.002
Gender			
Male	99 (54)	72 (59)	0.503
Female	83 (46)	51 (41)	
Race			
Black	17 (9)	15 (12)	0.383
Brown	52 (29)	41 (34)	
White	109 (61)	61 (50)	
Asian	2 (1)	3 (2)	
Indigenous	0 (0)	2 (2)	
Use of alcohol			
Never used	15 (8)	10 (8)	0.144
Ex-user	46 (25)	22 (18)	
User	121 (67)	91 (74)	
Smoking habit			
Non smoker	102 (56)	62 (50)	0.261
Smoker	80 (44)	61 (50)	
Metabolic syndrome (NCEP ATP III)			
Presence	62 (34)	78 (63)	< 0.001
Absence	120 (66)	45 (37)	
Diabetes mellitus			
Presence	25 (14)	45 (37)	< 0.001
Absence	157 (86)	78 (63)	
High cholesterol			
Presence	59 (33)	62 (51)	< 0.001
Absence	122 (67)	61 (49)	
Body mass index (kg/m^2)	26.6 (24.6-29.6)	29.2 (27.2-32.3)	< 0.001
Waist circumference (cm)	94.1 (87.5-100.0)	101.5 (94.1-107.5)	< 0.001
Systolic blood pressure (mmHg)	117 (108-127.5)	121.5 (113.5-131.2)	0.001
Diastolic blood pressure (mmHg)	75.5 (69.6-81.5)	79.5 (74.0-86.0)	< 0.001
Laboratory			
Fasting glucose (mg/dL)	99.28 (93.33-105.84)	104.24 (97.79-116.14)	< 0.001
HbA1c (mg/dL)	5.40 (5.1-5.8)	5.76 (5.3-6.2)	< 0.001
Insulin (mcUI/mL)	9.91 (7.1-13.9)	13.71 (10.5-19.32)	< 0.001
Total cholesterol (mg/dL)	199.6 (176.8-227.6)	196.2 (175.3-222.8)	0.636
Triglycerides (mg/dL)	107.8 (79.0-152.4)	137.15 (99.6-225.0)	< 0.001
HDL (mg/dL)	50.1 (42.9-60.1)	47.45 (42.0-54.2)	0.007
ALT (mg/dL)	25 (19-33)	30 (22.5-40)	0.001
AST (mg/dL)	24 (21-28)	24 (21-29.5)	0.276
GGT (mg/dL)	24 (18-35)	33 (23-51)	< 0.001
hs-CRP (mg/dL)	1.6 (0.8-3.5)	2.2 (1.4-4.7)	< 0.001

Uric acid (mg/dL)	5.4 (4.5-6.6)	6 (5.2-6.9)	< 0.001
Microalbuminuria (mg/dL)	0.50 (0.33-0.82)	0.51 (0.31-1.05)	0.265
Creatinine (mg/dL)	0.87 (0.77-0.97)	0.87 (0.77-0.97)	0.324
Sodium (mg/dL)	143 (141-144)	143 (141-144)	0.200
Potassium (mg/dL)	4.5 (4.2-4.7)	4.4 (4.2-4.7)	0.820

Data were presented as median (interquartile range) or count (percentage).
[1]Jonckheere-Terpstra for continuous variables and Cox-Mantel-Haenszel for categorical ones for trend between categories.
HbA1c: Hemoglobin A1c; HDL: High-density lipoprotein; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: Gamma-glutamyl transferase; hs-CRP: High sensitivity C-reactive protein.

Table 2 Non-alcoholic fatty liver disease-associated steatosis stratification accordingly to fatty liver serum biomarkers

	Mild (*n* = 182)	Moderate/severe (*n* = 123)	*P* value[1]
Fatty liver index	44.65 (26.71-67.91)	74.50 (54.08-88.16)	< 0.001
Hepatic steatosis index	36.66 (33.63-40.89)	41.22 (37.75-45.47)	< 0.001
Atherogenic index	0.35 (0.16-0.50)	0.44 (0.29-0.70)	< 0.001

Data were presented as median (interquartile range).
[1]Jonckheere-Terpstra test for trend between categories.

Table 3 Effect of high advanced glycation end product levels on the non-alcoholic fatty liver disease-associated steatosis severity

Variable	SE	OR (95%CI)	Sensitivity	Specificity	Accuracy
Crude model	0.47	2.64 (1.04-6.92)	0.62	0.61	0.61
Model 1	0.52	3.38 (1.24-9.98)	0.60	0.73	0.64
Model 2	0.55	3.34 (1.17-10.40)	0.71	0.68	0.69
Model 3	0.61	4.52 (1.44-16.28)	0.78	0.72	0.75
Model 4	0.62	4.66 (1.45-17.16)	0.77	0.77	0.77

Model 1: Adjusted for gender; Model 2: Additionally adjusted for gamma-glutamyl transferase; Model 3: Additionally adjusted for altered blood glucose (categorical ≥ 126, 110 ≤ 125); Model 4: Additionally adjusted for high cholesterol. High cholesterol was defined as use of medication to treat dyslipidemia or low-density lipoprotein cholesterol ≥ 130. OR: Odds ratio; CI: Confidence interval.

adjustment (0.83) would have the most clinical implications for predicting the severity of NAFLD-associated steatosis, with a sensitivity of 0.77, a specificity of 0.77, and an accuracy of 0.77, followed by model 3 (AUC = 0.81), model 2 (AUC = 0.79), model 1 (AUC = 0.72), and the crude model (AUC = 0.62) (Table 3, Supplementary Table 1, and Figure 3).

DISCUSSION

Addressing the specific mechanisms by which NAFLD progresses could potentially open new frontiers for preventive and monitoring strategies for liver diseases. In this study, we investigated the relationship between plasmatic AGE levels and severity of NAFLD-associated steatosis in a racially/ethnically diverse cohort at baseline of the ELSA-Brasil study. Steatosis stratification based on serum biomarker indexes, that is, FLI, HSI, and AI, was associated with the ultrasonography grade of NAFLD previously described[45-47], with higher values for moderate/severe group. Serum AGE levels were positively correlated with the steatosis stage; that is, AGE content was significantly higher in subjects with moderate/severe forms of steatosis compared to the mild form. Logistic regression analysis after adjusting for confounding variables showed that subjects with higher serum AGE content had a 4.6-fold increased chance

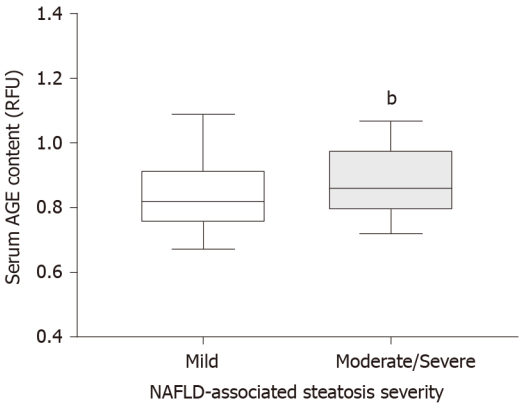


Figure 2 Boxplot showing the distribution of advanced glycation end product values in serum in the group of participants with mild steatosis and the group of participants with moderate/severe pooled steatosis. Data are shown as median ± inter-quartile range. [b]*P*< 0.001. AGE: Advanced glycation end product; RFU: Relative fluorescent units.

of having moderate or severe forms of steatosis when compared to that in those with low levels of serum AGEs. According to the results of the ROC analyses from the current study, AGEs could be a good marker of steatosis severity in patients with NAFLD and might be a potential biomarker in predicting NAFLD progression. Therefore, fluorescent AGEs could be a potential plasmatic biomarker for risk stratification of NAFLD accordingly to the severity of hepatic steatosis.

A proposed mechanism to explain why some patients with NAFLD progress to more severe forms of NAFLD is the multiple-hit hypothesis. In this hypothesis, the deposition of fat in the liver is the first hit, whereas the progression requires the involvement of other factors[48]. Emerging evidence highlighted that AGEs can act as a critical 'hit' being one factor that drives the progression from simple NAFLD to NASH and liver fibrosis[18,49]. Previously, the correlation of CML level with the clinical score of patients with liver cirrhosis showed a direct relationship with the severity of disease[50]. We and others have demonstrated increased deposition of AGEs in the liver of animals with NAFLD, MetS, and diabetes[35,41,50-57], which was associated with microcirculatory disturbances[53]. Furthermore, treatment with pyridoxamine, an AGE inhibitor, reduced the microcirculatory and metabolic alterations caused by NAFLD[53]. Using biopsies, Gaens *et al*[56] showed that Ne-(carboxymethyl) lysine (CML), as assessed by immunohistochemistry, was increased in the liver of obese individuals who underwent bariatric surgery, and AGE levels were associated with the grade of hepatic steatosis and inflammation, suggesting that AGEs could contribute to NAFLD progression. Furthermore, incubation of human hepatic stellate cells with AGEs increased the expression of fibrotic and cell proliferation markers, suggesting that these compounds can contribute to the development of NASH[58]. Mechanistically, AGE formation may result in loss of hepatocyte function, while intracellular accumulation of AGEs causes cell death[59]. Moreover, the interaction of extracellular AGEs with its receptor RAGE in hepatocytes[60] promotes inflammation, a characteristic feature of NAFLD progression to NASH[61]. In addition, fatty acids stimulate CML accumulation in hepatocytes and subsequently elicit inflammatory reactions *via* RAGE activation[56].

Although some studies have highlighted the involvement of AGEs in liver diseases, only two previous studies assessed the correlation between serum AGEs and the severity of NAFLD. The study by Hyogo *et al*[34] showed that AGE levels were elevated in NASH patients compared to individuals with simple steatosis. Świderska *et al*[62] reported that AGEs can be used as a biomarker to differentiate between patients with minimal *vs* moderate steatosis, but in a small cohort (early NAFLD n = 29 and advanced NAFLD n = 38). We addressed the AGE levels in subjects with mild and moderate/severe steatosis in a larger cohort. Presently, we assessed AGE levels by fluorescent spectroscopy, while the study by Hyogo *et al*[34] used ELISA. Fluorescence spectroscopy is an easy, rapid, and cost-effective method that has been previously proposed as a reliable tool that can be used to distinguish patients with type 1 and 2 diabetes from healthy subjects[63,64]. Recently, Heidari *et al*[65] showed that serum levels of AGEs increase progressively with increasing duration of diabetes, and proposed the monitoring of fluorescent AGEs as an estimation tool for diabetes chronicity.

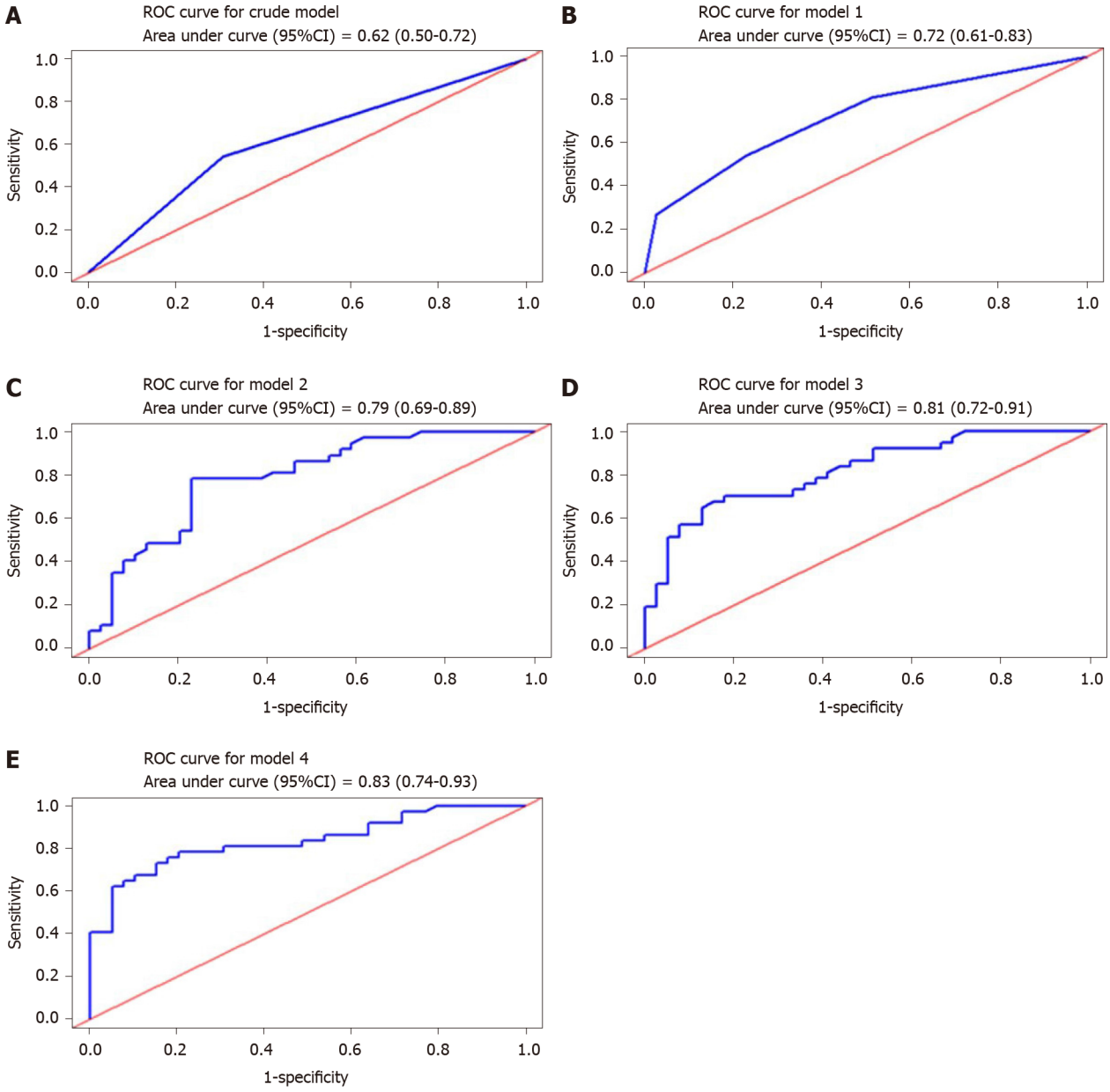


Figure 3 Receiver operating characteristic curves and corresponding areas under the curve of models to predict non-alcoholic fatty liver disease-associated steatosis. ROC: Receiver operating characteristic; CI: Confidence interval.

Along with increasing NAFLD-associated steatosis severity, increased AGE is associated with a concomitant worsening of the metabolic profile, namely MetS, diabetes mellitus, cholesterol levels, BMI, waist circumference, blood pressure, and lipid and glycemic profile[66-70]. In our study, the results of logistic multiple regression analysis showed that, as well as AGEs, altered fasting blood glucose and high cholesterol levels were associated with the severity of steatosis in NAFLD. The risk of severe steatosis in individuals with altered fasting blood glucose and high cholesterol was 6.730 and 3.490 times higher than that in subjects with a normal range of blood glucose and cholesterol, respectively. Although the precise pathological process has not been elucidated, it has been widely accepted that excess triglyceride accumulation in the liver is a prerequisite for NAFLD development. Triglyceride showed a great correlation with NAFLD in both epidemiologic studies[71] and pre-clinical research[72]. The progression of NAFLD results from an imbalance between lipid uptake and lipid disposal and eventually causes oxidative stress and hepatocyte injury. Excess lipid accumulation may result in impaired insulin signaling through cell autonomous mechanisms or through the induction of inflammation and the subsequent production of inflammatory cytokines by macrophages, which impair insulin action[73]. Increasing evidence suggests that subjects with hyperglycemia resulting from defects in insulin secretion, insulin action, or both have a particularly

high risk for NASH, with varying degrees of liver fibrosis[74,75]. Insulin resistance may cause inflammation in the adipose tissue, which triggers the acceleration of lipolysis, resulting in increased free fatty acid export to the liver, leading to the accumulation of fat in the liver and consequently NAFLD[48,76]. It has already been demonstrated that AGE formation is accelerated under a hyperglycemic state, playing an important role in the pathogenesis of NAFLD and complications of diabetes[77,78]. Lipid peroxidation is also known to be involved in the generation of AGEs, and the glycation of lipids results in the formation of AGEs, which are related to triglyceride or HDL-C levels[79].

The current study has limitations that should be mentioned. First, it was not possible to distinguish people with NAFLD from healthy controls based on serum fluorescent AGE concentrations. Moreover, given the cross-sectional design of the study, no causal relations can be established. The sample size is higher than others in the field, but the sample size of the severe form of NAFLD-associated steatosis remains small, probably due to the study population being formed by healthy employees. Thus, the associations found here may be even greater in the general population. This might have limited our results to fully explore the relationship between glycation products and disease stages. As this was a single-center study, results and glycation levels should be extrapolated to other populations carefully.

CONCLUSION

In conclusion, this cross-sectional analysis of the ELSA-Brasil cohort baseline study showed that high serum AGE content was associated with severe forms of steatosis, with high serum AGE levels being an important risk factor for NAFLD-associated steatosis severity. In addition, serum AGE content was positively correlated with the severity of steatosis. These data suggest that AGEs might be a potential plasmatic biomarker for NAFLD stratification accordingly to hepatic steatosis. Our findings strengthen the involvement of AGE in NAFLD pathogenesis. These findings highlight the importance of including the evaluation of AGE status as a part of health examinations and may help health policy-makers prevent or delay NAFLD among the population.

ARTICLE HIGHLIGHTS

Research background

Non-alcoholic fatty liver disease (NAFLD) is considered the hepatic manifestation of metabolic syndrome and it affects about 25% of the adult population, and can progress to hepatocellular carcinoma, death, and/or liver transplantation. The underlying mechanisms that account for disease progression are still not fully understood due to its complexity.

Research motivation

Liver diseases are associated with the excess formation of advanced glycation end products (AGEs), which induce tissue inflammation and oxidative damage. However, the trend of oxidative marker levels according to NAFLD severity is unclear.

Research objectives

We aim to understand whether NAFLD-associated steatosis severity was associated with serum AGE levels in the baseline data from the Brazilian Longitudinal Study of Adult Health (ELSA-Brasil) study to address the specific pathophysiological mechanisms underlying NAFLD association with AGEs in a large and mixed population cohort.

Research methods

NAFLD-associated steatosis severity was classified by ultrasound hepatic attenuation: mild and moderate/severe pooled. The measurement of serum fluorescent AGE concentrations was based on spectrofluorimetric detection. Serum AGE content and clinical and laboratory characteristics of the participants were compared between groups. The correlation between serum AGE levels and the grade of steatosis was analyzed. Logistic regression analysis was used to investigate the relationship between

serum AGE levels and NAFLD-associated steatosis severity. A P value < 0.05 was considered statistically significant.

Research results

According to hepatic steatosis grade in NAFLD, from mild to moderate/severe, individuals with the most severe forms of steatosis had a higher incidence of metabolic syndrome, diabetes mellitus, and high cholesterol levels. Moreover, individuals with increasing severity of NAFLD-associated steatosis presented increasing waist circumference, body mass index, systolic and diastolic blood pressure, fasting blood glucose, glycated hemoglobin, insulin, triglycerides, alanine aminotransferase, gamma-glutamyl transferase, C-reactive protein, and uric acid levels and lower high-density lipoprotein. Higher serum AGE content was present in the moderate/severe group of individuals than in the mild group. In addition, the serum AGE levels were correlated with the steatosis grade in the overall sample. Logistic regression analysis, after adjusting for confounding variables, showed that subjects with higher serum AGE content had a 4.6-fold increased chance of having moderate or severe forms of NAFLD-associated steatosis when compared to low levels of serum AGEs.

Research conclusions

Steatosis severity in NAFLD patients was associated with serum AGE levels, thereafter AGEs could be a good marker of NAFLD stratification accordingly to steatosis grade, strengthening the involvement of AGE in NAFLD pathogenesis.

Research perspectives

Plasmatic fluorescent AGE quantification by spectroscopy could be a promising alternative method to monitor progression from mild to severe forms of NAFLD accordingly to the severity of hepatic steatosis.

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CASE REPORT

Autoimmune enteropathy and primary biliary cholangitis after proctocolectomy for ulcerative colitis: A case report and review of the literature

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Author contributions: Zhou QY drafted the manuscript; Zhou WX collected the pathological data and interpret the histological examination; Zhou QY, Zheng WY, and Li Y contributed to clinical data collection and follow up; Wu B and Sun XY performed the operation; Li Y and Qian JM critically revised the manuscript; all authors have read and approved the final manuscript.

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Abstract

BACKGROUND

Autoimmune enteropathy (AIE) and primary biliary cholangitis (PBC) are both immune-mediated diseases. AIE or PBC complicated with ulcerative colitis (UC) are rare. There are no cases of AIE and PBC diagnosed after proctocolectomy for UC reported before, and the pathogenesis of these comorbidities has not been revealed.

CASE SUMMARY

A middle-aged woman diagnosed with UC underwent subtotal colectomy and ileostomy due to the steroid-resistant refractory disease, and a restorative proctectomy with ileal pouch-anal anastomosis and proximal neoileostomy was postponed due to active residual rectal inflammation in January 2016. A few months after the neoileostomy, she began to suffer from recurrent episodes of watery diarrhea. She was diagnosed with postcolectomy enteritis and stoma closure acquired a good therapeutic effect. However, her symptoms of diarrhea relapsed in 2019, with different histological features of endoscopic biopsies compared with 2016, which showed apoptotic bodies, a lack of goblet and Paneth cells, and villous blunting. A diagnosis of AIE was established, and the patient's stool volume decreased dramatically with the treatment of methylprednisolone 60

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mg/d for 1 wk and tacrolimus 3 mg/d for 4 d. Meanwhile, her constantly evaluated cholestatic enzymes and high titers of antimitochondrial antibodies indicated the diagnosis of PBC, and treatment with ursodeoxycholic acid (16 mg/kg per day) achieved satisfactory results.

CONCLUSION

Some immune-mediated diseases may be promoted by operation due to microbial alterations in UC patients. Continuous follow-up is essential for UC patients with postoperative complications.

Key Words: Autoimmune enteropathy; Primary biliary cholangitis; Ulcerative colitis; Proctocolectomy; Bacterial translocation; Case report

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Core Tip: This is the first case report of autoimmune enteropathy and primary biliary cholangitis complicated with ulcerative colitis, and the female patient in this case suffered from a tortuous process of diagnosis and treatment. It is speculated that a microbial shift and subsequent immune response are involved in the pathogenesis of these comorbidities, and surgery may facilitate the progression of coexisting diseases. Understanding the progression of these diseases may help with their early recognition and treatment.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) affecting the colon and rectum. Dysregulated immune responses to microbial changes and epithelial barrier defects are reported to contribute to the pathogenesis of UC. Small bowel involvement in UC is usually characterized by backwash ileitis or pouchitis[1]. Diffuse and severe enteritis occurring after colectomy for UC, which is referred to as postcolectomy enteritis, has been sporadically reported in the literature[2-5]. The pathogenesis and prognosis remain obscure. A case of postcolectomy enteritis previously reported by our team[3] recently presented recurrent large-volume watery diarrhea and typical pathological features of autoimmune enteropathy (AIE) along with high titers of antimitochondrial antibodies (AMAs) and increased cholestatic enzymes. The association of UC with primary sclerosing cholangitis (PSC) has been commonly reported[6], whereas UC with primary biliary cholangitis (PBC) has been rarely reported, and the underlying pathogenesis may be different. In this report, we present a rare case of progressive AIE and PBC after colectomy for UC and propose a hypothesis to explain the potential associations of these coexisting diseases.

CASE PRESENTATION

Chief complaints

A 54-year-old woman presented to the Emergency Department complaining of large-volume watery diarrhea.

History of present illness

The patient was diagnosed with moderate to severe extensive UC at the age of 49 in December 2014. Subtotal colectomy and ileostomy were performed in February 2015 due to steroid-resistant refractory disease. The UC diagnosis was pathologically

confirmed in resected specimens without ileitis. Her general condition improved greatly after ileostomy. A restorative proctectomy with ileal pouch-anal anastomosis and proximal neoileostomy was postponed until January 2016 due to active residual rectal inflammation. Within 3 mo after the neoileostomy, the patient noticed that watery stool from the stoma gradually increased up to 3-4 L per 24 h. She was admitted to our hospital for hypovolemic shock, electrolyte and acid-base disturbances, and acute kidney injury. Laboratory tests showed numerous leucocytes in liquid stool but negative pathogenic culture and *Clostridium difficile* toxin results. Her stoma output did not respond to empiric antibiotics but decreased significantly with IV hydrocortisone (50 mg q6h). Endoscopy showed a normal gross appearance of the stomach, duodenum, and prestomal ileum (Figure 1). Pathological examinations of the endoscopic biopsy from the duodenum and prestomal ileum revealed enteritis, which manifested as moderate villous atrophy, decreased goblet cells, cryptitis, and destroyed crypts, without clear apoptotic bodies or intraepithelial lymphocytosis (Figure 2). Postcolectomy enteritis was confirmed, and the decision to close the stoma was made by our multidisciplinary team to re-establish fecal stasis. Two months after stoma closure, her stool volume gradually decreased to less than 1 L/d, and azathioprine 50 mg/d was added as maintenance treatment during prednisone tapering. Follow-up endoscopy and biopsy histology in March 2018 revealed a normal mucosal appearance and the recovery of villous atrophy, cryptitis, and inflammation in the lamina propria. She made the decision to stop azathioprine in 2018 on her own. In November 2019, she presented with recurrent hypovolemic shock secondary to large-volume watery diarrhea under no obvious predisposing causes, which had been worsened to 2-3 L liquid stool every 24 h in the last month. She also mentioned poor appetite and a body weight loss of 8 kg within a month.

History of past illness

The patient had a past medical history of poliomyelitis.

Personal and family history

Unremarkable.

Physical examination

The patient was in serious weakness with cold limbs when admitted. Her vital signs were as follows: Temperature was 36.1 °C, heart rate was 109 bpm, respiratory rate was 20 breaths/min, blood pressure was 87/66 mmHg, and oxygen saturation in room air was 99%. The clinical physical examination revealed hyperactive bowel sounds 7-8 times/min, and no abdominal tenderness during palpation.

Laboratory examinations

Blood analysis revealed hemoglobin 181 g/L, platelets 274 × 10^9/L, white blood cells 11.63 × 10^9/L, neutrophils 68.7%, and lymphocytes 20.6%. Blood biochemical values were abnormal, with alanine aminotransferase (ALT) 22 U/L, total bilirubin 22.4 μmol/L, albumin 48 g/L, creatinine 206 μmol/L, urea 10.15 mmol/L, serum potassium 5.1 mmol/L, and serum sodium 128 mmol/L. Her arterial blood gas parameters were: pH 7.55, partial pressure CO_2 23 mmHg, partial pressure O_2 113 mmHg, bicarbonate 20.1 mmol/L, base excess 0.3 mmol/L, and lactic acid 3.1 mmol/L. Serum C-reactive protein was increased at 24.55 mg/L (normal range ≤ 8 mg/L) and erythrocyte sedimentation rate at 34 mm/h. Routine stool test showed 6-8 white blood cells per high power field and occult blood was noted. Stool pathogen screening was negative except for *Candida albicans* in stool culture.

Imaging examinations

No significantly abnormal signs were found on abdominal and pelvic computed tomography (CT). Endoscopy revealed an intact mucosa with diffuse villous atrophy in the duodenum, ileum, and ileal pouch (Figure 3). Histopathological findings of biopsies showed apoptotic bodies, a lack of goblet and Paneth cells, and villous blunting, which were not prominent in 2016 (Figure 4).

FINAL DIAGNOSIS

The principal clinical manifestation of AIE was refractory diarrhea with no response to dietary modification. Characteristic histological features included villous blunting or

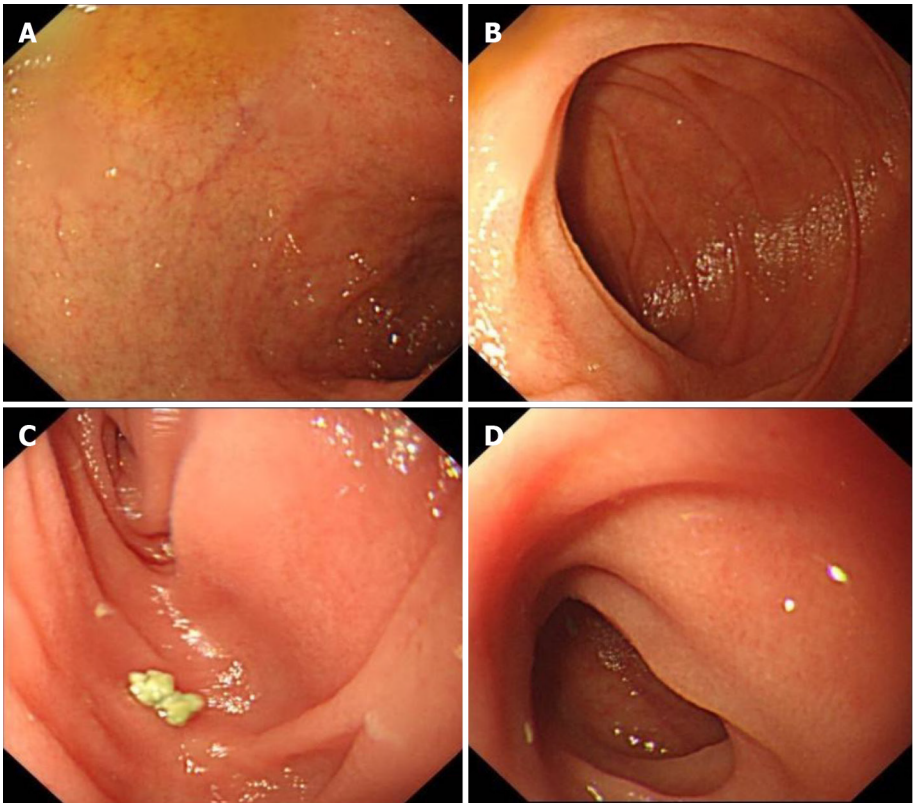


Figure 1 Endoscopic images at the onset of postcolectomy enteritis in 2016. A: Gastroscopy showing a normal duodenal bulb; B: Gastroscopy showing a normal descending duodenum; C and D: Endoscopy with loop ileostomy showing a normal appearance of the neoileum.

atrophy, apoptotic bodies, and lymphocytic infiltration in the crypt epithelium. The absence of goblet and Paneth cells also supported a diagnosis of AIE[7]. Given the typical clinical and histological features strongly supporting AIE and no evidence for other diseases causing villous atrophy and mucosal inflammation in this case, a diagnosis of AIE was established. As the sensitivity and specificity of AMA-M2 for PBC diagnosis are both 90%-95%[8], positive AMA-M2 made the diagnosis of PBC definitive. Despite having no signs of extrahepatic biliary obstruction on magnetic resonance cholangiopancreatography, small duct PSC could not be excluded completely due to a lack of histologic evidence. A previous case report showed the overlap of PBC and small duct PSC[9], indicating that typical onion-skin lesions in biliary ducts, with concentric fibrosis, can be seen in small duct PSC with MRI negativity. As a result, liver biopsy is needed to confirm the diagnosis of PBC, if possible.

TREATMENT

Supportive treatments with intravenous infusions, vasoactive drugs, and regulating electrolyte disturbances were performed timely to maintain the stable vital signs. Methylprednisolone 60 mg/d was given intravenously for 1 wk, and tacrolimus 3 mg/d (target blood concentration 3-10 ng/mL) was given to maintain the treatment response when steroids were tapered. As for PBC, ursodeoxycholic acid 16mg/kg per day was prescribed.

OUTCOME AND FOLLOW-UP

The patient's vital signs became stable under sufficient supportive treatments. After treatment with methylprednisolone 60 mg/d for 1 wk, her stool volume gradually decreased to less than 1.5 L/d. After taking tacrolimus for 4 d, the stool volume decreased to approximately 0.5 L/d and solid components could be seen in the liquid stool. The levels of ALT, aspartate aminotransferase, alkaline phosphatase, gamma-

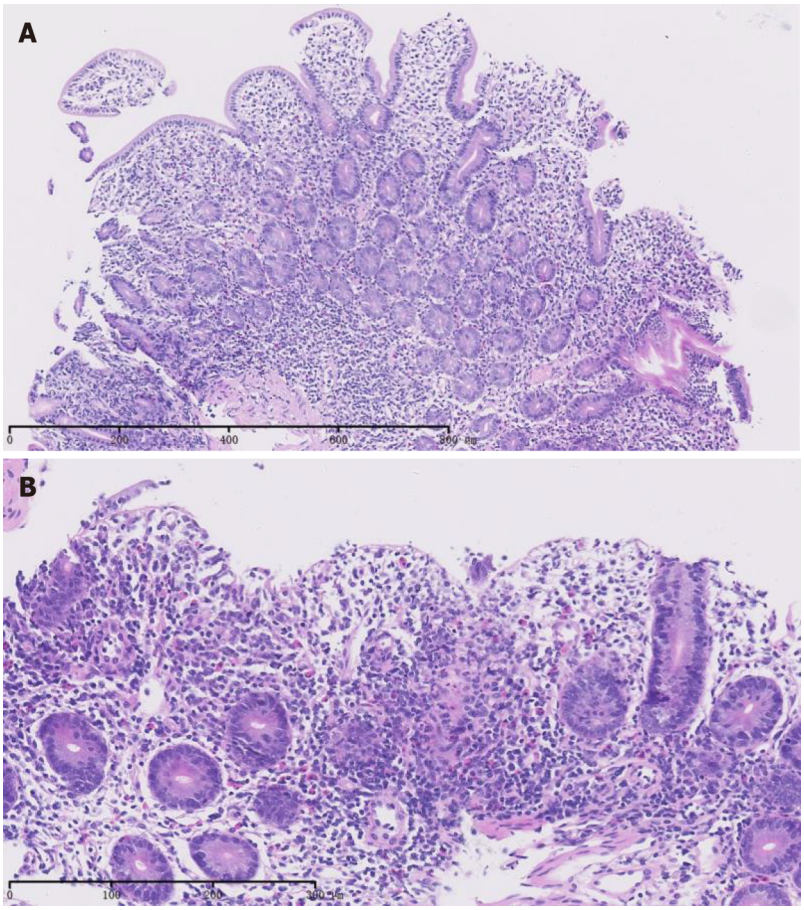


Figure 2 Pathological findings at the onset of postcolectomy enteritis in 2016. A: The biopsy from the duodenum showed chronic inflammation, moderate villous atrophy, and decreased goblet cells (× 40); B: Active inflammation, cryptitis, and destroyed crypts were observed in some areas (× 100).

glutamyl transferase, and serum total bilirubin decreased gradually compared with the peak levels (109 U/L to 67 U/L, 87 U/L to 30 U/L, 404 U/L to 87 U/L, 659 U/L to 137 U/L, and 161.8 μmol/L to 20.3 μmol/L, respectively).

DISCUSSION

We searched the PubMed and Embase databases for case reports published in English or with an English abstract before June 22, 2020, with the keywords "ulcerative colitis", "autoimmune enteropathy", and "primary biliary cholangitis" (or "primary biliary cirrhosis"). There is only one case of AIE in a UC patient[10] and 20 cases of PBC in UC patients[11-13]. Therefore, our patient is the first case of UC complicated with AIE and PBC after colectomy.

This patient presented similar watery diarrhea in 2019 as in 2016, and the pathological features of the small intestine progressed. The diagnosis of postcolectomy enteritis was based on clinical manifestations of diffuse, superficial mucosal inflammation of the small bowel after colectomy in a UC patient, and the underlying pathogenesis remains unknown. In this case, with recurrent enteritis, typical pathological features of AIE were noticed during relapse, and we speculate that this progression was mediated by the same pathogenic mechanisms. We review the 23 previous cases of postcolectomy enteritis in UC (from an English database, including two cases in Japanese with English abstracts)[2,3] and summarize the histological characteristics of small intestinal biopsies[2,4,5,14-25] (Table 1). A large proportion of these cases exhibited typical features of UC, with inflammatory infiltration of the lamina propria by lymphocytes, plasma cells, monocytes, or a few neutrophils found in 19 cases and cryptitis or crypt abscess in 9 cases. Typical features of AIE, including apoptotic bodies and decreased goblet cells (3 and 1 cases, respectively), were also reported in these patients[4,5]. Above all, we propose a hypothesis that these pathological features are present at different stages in a dysregulated immune

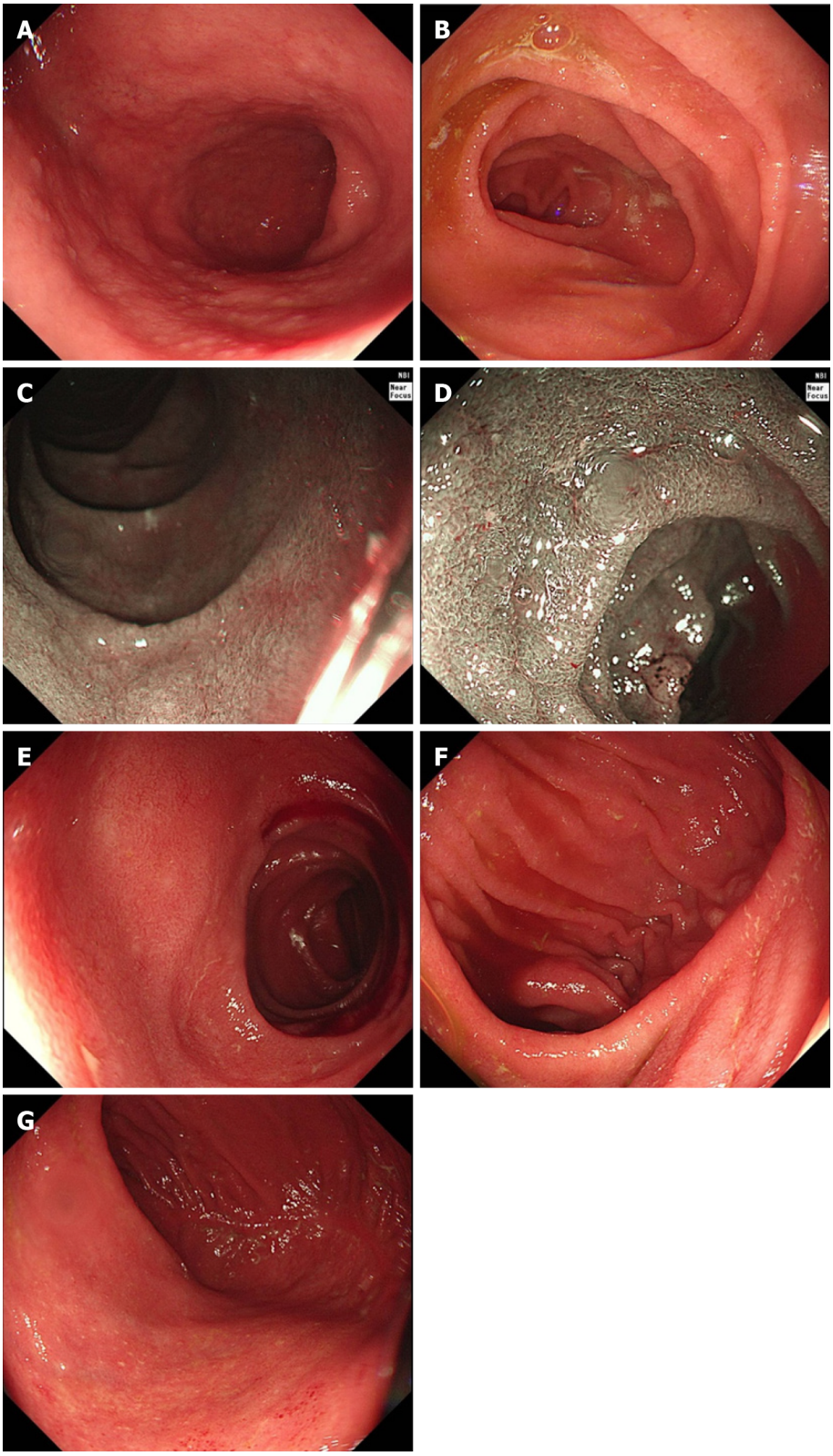


Figure 3 Endoscopic images at the onset of autoimmune enteropathy in 2019. A: Gastroscopy showing diffuse villous atrophy in the mucosa of the duodenal bulb; B and C: Gastroscopy showing diffuse villous atrophy in the mucosa of the descending duodenum; D and E: Enteroscopy showing diffuse villous atrophy in the mucosa of the ileum; F and G: Enteroscopy showing diffuse villous atrophy in the mucosa of the ileal pouch.

condition triggered by a microbial shift after colectomy. It is possible that three cases of postcolectomy enteritis with features of AIE (apoptotic bodies)[4,5] may progress to AIE during follow-up because these cases presented with severe clinical manifestations similar to our patient, with one of them even worse (dying of multiorgan failure).

Table 1 Summary of cases of postcolectomy enteritis from the English databases

Ref.	Age[1]/sex	Extent of UC	Onset of enteritis[2]	Extent of enteritis	Histological characteristics of biopsies
Corporaal *et al*[2]	23/M	Extended left sided	2 wk after colectomy	J, I	Marked apoptosis of epithelial cells, lamina propria with lymphoplasma cellular infiltrate with eosinophilic and neutrophilic granulocytes
Corporaal *et al*[2]	61/M	Pancolitis	1 mo after colectomy	S, D, J, I	Diffuse active chronic inflammatory infiltrate with (sub) total villous atrophy (D), an increase in the apoptosis of crypt epithelial cells (I), and dense active chronic inflammation (S)
Gooding *et al*[14]	52/M	Pancolitis	116 d after colectomy	D, I	Infiltration of the lamina propria by lymphocytes, plasma cells, and neutrophils; cryptitis and small numbers of neutrophils within the upper-crypt lamina and on the surface (D); shortening and irregularity of villi; mild, diffuse, chronic inflammatory cell infiltration; and mild activity with cryptitis (I)
Ikeuchi *et al* [15]	29/F	Left sided	6 yr after colectomy	S, D, I	Severe infiltration of inflammatory cells in the mucosa (I, S) and crypt abscess in the mucous glandules (S), dense, acute, and chronic inflammatory infiltrates accompanied with cryptitis (D)
Kawai *et al* [16]	Young (unknown)/M	Pancolitis	12 yr after colectomy	D	Lymphoplasmacytic infiltration of the duodenal mucosa without granuloma formation
Kawai *et al* [16]	Unknown/M	Unknown	2 yr after colectomy	I	Unknown
Rubenstein *et al*[17]	38/M	Pancolitis	Immediately after colectomy	D, J, I	Diffuse mucosal inflammation with the distortion of the crypt architecture and plasmacytosis
Nakayama *et al*[18]	41/M	Pancolitis	3.5 yr after colectomy	D, I	Inflammatory infiltration by neutrophils, eosinophils, lymphocytes, and plasma cells
Terashima *et al*[19]	30/M	Left sided	7 mo after colectomy, immediately after closure of ileostoma	D	Severe lymphoplasmacytic infiltration withcryptitis
Valdez *et al* [20]	5/F	Pancolitis	17 mo after colectomy	D	Diffuse mucosal inflammation, cryptitis, and architectural distortion
Valdez *et al* [20]	17/F	Pancolitis	9 mo after colectomy	D, I	Diffuse mucosal inflammation, cryptitis, and architectural distortion
Valdez *et al* [20]	58/F	Pancolitis	6 mo after colectomy	D, J	Typical microscopic features of UC (no other details)
Valdez *et al* [20]	17/M	Pancolitis	8 d after colectomy	D, I	UC-like active, diffuse duodenitis and ileitis (no other details)
Annese *et al* [21]	37/M	Pancolitis	1 mo after colectomy	D, J, I	Architectural disarray and dense inflammatory infiltrate extending beyond the muscularis mucosae, mainly comprised of plasma cells and lymphocytes (D, J). Distorted villi and a heavily inflamed lamina propria with mononuclear cell inflammatory infiltrate (I)
Feuerstein *et al*[4]	56/F	Left sided	26 d after colectomy	D, J, I	Severe, active enteritis with ulceration and prominent epithelial cell, apoptotic debris
Nakajima *et al*[5]	25/F	Pancolitis	Immediately after colectomy	D, J, I	Infiltration of inflammatory cells in the mucosa and decreased goblet cells
Hoentjen *et al*[22]	23/M	Pancolitis	3 mo after colectomy	S, D, I	Severe active inflammation with significant neutrophil infiltration in the epithelium and lamina propria
Hoentjen *et al*[22]	24/F	Pancolitis	4 mo after colectomy	S, D, I	Mixed cell infiltrates in the lamina propria, including a prominent component of plasma cells and clusters of neutrophils, negative for crypt cell apoptosis and decreased goblet cells
Hoentjen *et al*[22]	27/M	Pancolitis	25 yr after colectomy	S, D, I	Active inflammation with prominent eosinophils in the lamina propria (S, D) and remarkable architectural distortion (I)
Hoentjen *et al*[22]	57/M	Pancolitis	27 mo after colectomy	S, D, I	Diffuse and active *Helicobacter pylori*-negative gastritis, duodenitis and ileitis (no other details)
Uchino *et al* [23]	19/M	Pancolitis	12 d after colectomy	S, D, I	Neutrophil infiltration and crypt abscess
Rush *et al* [24]	43/F	Pancolitis	3 mo after colectomy	S, D, J, I	Moderate to marked active chronic inflammation with cryptitis
Rosenfeld *et al*[25]	47/F	Pancolitis	4 mo after colectomy	D, J	Moderate to severe active inflammatory changes in the small bowel mucosa with cryptitis

[1]Age at the onset of postcolectomy enteritis.
[2]Interval between colectomy and the onset of enteritis symptoms. S: Stomach; D: Duodenum; J: Jejunum; I: Ileum; UC: Ulcerative colitis; F: Female; M: Male.

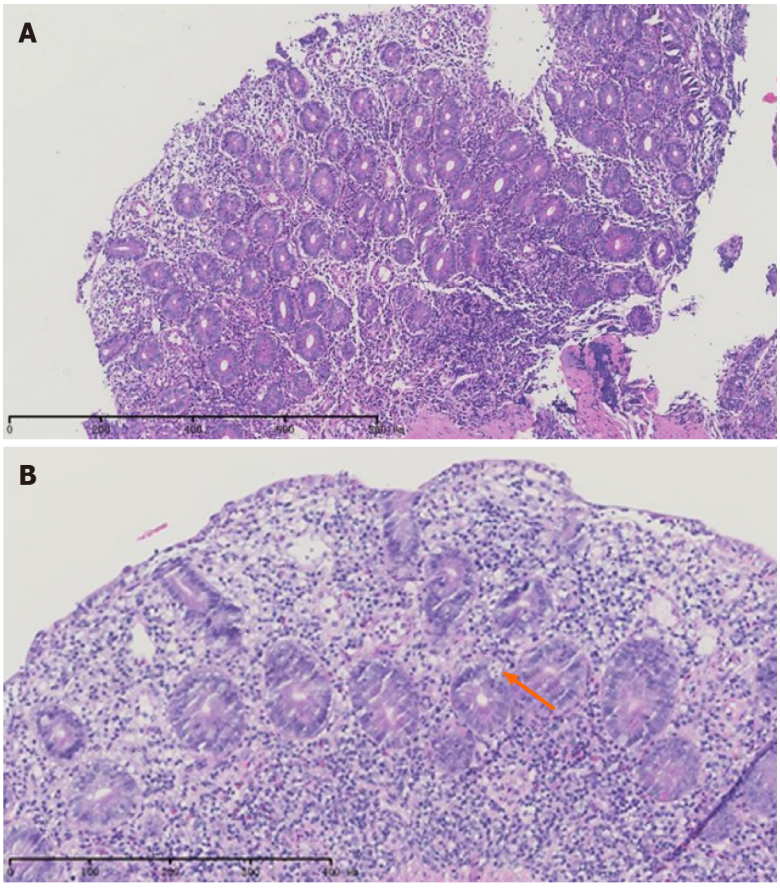


Figure 4 Pathological findings at the onset of autoimmune enteropathy in 2019. The biopsy showed prominent chronic inflammation, flat villi, and a lack of goblet and Paneth cells (A), and apoptosis bodies were found (arrow) (B).

The nature of the relationship between PBC and UC is unexplained. Immune-pathogenic mechanisms may be implicated in the pathogenesis of UC complicated with autoimmune hepatobiliary disorders[26]. Lymphocytic infiltration of portal tracts and the presence of circulating antibodies that react with bile ducts[7,27] suggest that the two diseases share common immunological pathways. Our patient initially suffered from refractory and progressive disease after colectomy in 2016, which suggests that surgery may be a potential factor that promotes disease progression. A widely accepted hypothesis regarding the pathogenesis of IBD indicates that the mucosal immune system exhibits an aberrant response towards luminal antigens, including commensal bacteria[28,29]. Meanwhile, some researchers have proposed that molecular mimicry may be a potential pathogenic mechanism underlying immune-mediated biliary damage. Antibodies that bind to the mitochondrial E2 subunit of the pyruvate dehydrogenase complex (PDC-E2) also cross-react with conserved bacterial proteins, including microbes found on mucosal surfaces and in the feces[26]. Such bacterial mimics of PDC-E2 were believed to trigger AMA-M2 formation in the early stage of PBC pathogenesis[29]. Another "leaky gut" hypothesis proposed to explain the disease process in PSC inspired us to investigate the pathogenesis of hepatobiliary disorders. The disruption of bowel permeability may lead to bacterial translocation and bile colonization and subsequently activate the inflammatory response and fibrosis in the liver by activating cholangiocytes[30]. A case report described a female patient with UC who developed PBC after proctocolectomy[31]. Whether the occurrence of AIE and PBC after proctocolectomy in this patient was accidental or predestined based on the genetic, microbial, and immune background is worthy of further investigation.

CONCLUSION

We have reported a patient with UC complicated with AIE and PBC and hypothesized the possible underlying pathogenesis. Although there is insufficient clear evidence of the mechanism, this case offers us a new explanation for the spectrum of immune-mediated diseases. Proctocolectomy may promote the progression of UC due to microbial alterations, leading to complications in other target organs. For UC patients with postoperative complications, continuous follow-up is essential for early recognition and comprehensive treatment.

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REVIEW

Diabetes and pancreatic cancer: Exploring the two-way traffic

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Author contributions: Roy A, Sahoo J and Mohan P did the literature search; Roy A wrote the first draft of the review; Sahoo J and Kamalanathan S conceptualized the work, supervised the writing, gave intellectual inputs, and critically revised the manuscript; Naik D, Mohan P and Kalayarasan R gave intellectual inputs and critically revised the manuscript.

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Abstract

Pancreatic cancer (PC) is often associated with a poor prognosis. Long-standing diabetes mellitus is considered as an important risk factor for its development. This risk can be modified by the use of certain antidiabetic medications. On the other hand, new-onset diabetes can signal towards an underlying PC in the elderly population. Recently, several attempts have been made to develop an effective clinical tool for PC screening using a combination of history of new-onset diabetes and several other clinical and biochemical markers. On the contrary, diabetes affects the survival after treatment for PC. We describe this intimate and complex two-way relationship of diabetes and PC in this review by exploring the underlying pathogenesis.

Key Words: Chronic pancreatitis; Diabetes; New onset diabetes; Pancreatic adenocarcinoma; Pancreatic cancer; Type 3c diabetes

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Core Tip: Type 2 diabetes mellitus can increase the risk of pancreatic cancer (PC) and certain antidiabetic medications can modify this risk. New onset diabetes in

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combination with other clinical and biochemical markers can serve as an effective screening tool for PC. On the contrary, the glycaemic status affects the treatment outcome of PC. More awareness among clinicians is required about the two-way relationship between diabetes mellitus and PC.

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INTRODUCTION

Pancreatic cancer (PC) is one of the few malignancies associated with a dismal prognosis. Its incidence is on the rise and is one of the leading causes of cancer-related death worldwide[1]. Similarly, type 2 diabetes mellitus (T2DM) accounts for a significant morbidity and mortality owing to a global increase in its incidence. Its prevalence is predicted to reach up to 700 million by 2045[2]. Longstanding diabetes has been regarded as a modest risk factor for PC. On the other hand, new-onset diabetes mellitus (NOD), especially after the 5th decade of life is often observed as a harbinger of an underlying PC. There is also a simultaneous increase in obesity worldwide, which plays a key role in development of both T2DM and PC[3]. Thus, this surge in diabetes and obesity prevalence may eventually increase the risk of PC in a significant number of population in near future[4].

Diabetes and PC have a multifaceted relationship. There are different types of diabetes as per the American Diabetes Association but two types of diabetes, namely T2DM and type 3c diabetes, merit attention in relation to PC[5]. T2DM is a chronic non-communicable disease characterised by hyperglycaemia resulting from the defective insulin secretion due to progressive beta cell dysfunction in the face of ongoing insulin resistance (IR)[5]. The diabetes associated with different exocrine pancreatic disorders is known as type 3c diabetes. The duration of DM has an important relationship with development of PC. However, the time duration cut-offs to define different types of diabetes are arbitrary and are varied. The time duration taken to define NOD in the context of PC is between 2-3 years[6]. On the contrary, when the diabetes is present for more than 2-3 years before the diagnosis of PC, it is considered as a long-standing T2DM. However, differentiating between this two entities is very difficult in a given subject of PC, since many patients of T2DM have a long asymptomatic undiagnosed period[5].

T2DM can have an impact on the outcome of different treatment modalities of the PC. Moreover, different drugs used for treating T2DM can affect the risk of PC as well. Metformin has gained particular attention in this context. In the appropriate clinical context, a recent worsening glycaemic profile requiring insulin might point towards the development of PC in the elderly diabetes subjects. The main obstacle in the diagnosis of PC in DM is to identify the candidates to be screened as routine evaluation for PC is not recommended in them. Ongoing research in identifying the screening population based on clinical characteristics and biomarkers and developing different models based on the combination of such parameters continues. In this background, we aim to review the current literature for unfolding the complex but intricate relationship between diabetes and PC.

SEARCH STRATEGY

The PubMed search was carried out for relevant articles by three authors (AR, JS, PM). The references of the pertinent articles were also searched for additional appropriate studies. The keywords and combinations included in the search were: 'diabetes'; 'new onset diabetes'; 'pancreatic cancer-related diabetes'; 'pancreatic cancer' and 'diabetes'; 'new onset diabetes' and 'pancreatic cancer'; 'long term diabetes' and 'pancreatic cancer'; 'pancreatic ductal adenocarcinoma' and 'diabetes'; 'metformin' and 'pancreatic cancer' and 'diabetes'; 'Type 3c diabetes' and 'pancreatic cancer'. The

search was restricted to only English literature and predominantly focused on the recent evidence. The appropriate articles to be included in this review were selected by SK, DN and RK.

RISK OF PC IN LONG-STANDING DIABETES MELLITUS

The evidence of association between NOD and the PC is consistent (see below); however, the evidence for risk of development of pancreatic ductal adenocarcinoma (PDAC) in long-standing diabetes is mixed. PDAC is the most common form of PC. Moreover, the risk is cumulative and is in continuum with the fasting blood glucose levels and the risk consistently increases from normal glucose tolerance to prediabetes to diabetes[7].

The increased risk of PC in long-standing T2DM has been suggested across different population of the world, including Asians[8-10]. A recent report involving a large population (n = 112818 females and 46207 males respectively) over 30 years of cumulative exposure showed an increased risk of PDAC with long-standing diabetes over time (age-adjusted hazard ratio [HR] 2.16 [95%CI: 1.78-2.60])[11]. Another recently published meta-analysis also suggested an increased PC related mortality with T2DM (relative risk [RR] 1.67; [95%CI: 1.30-2.14])[12].

The summary of the evidence suggests that the reported RR for developing PDAC in long-term diabetes is modest and varies between 1.4-2.1[8,13]. The risk may persist even after adjustment for obesity and smoking, two important and independent risk factors for PDAC[14]. Additionally, PDAC risk is significantly more in NOD and although the risk reduces subsequently, it may remain significant as the duration of the diabetes gets longer as per few meta-analysis[13,15]. However, a 2015 summary review of the available meta-analysis questioned the robustness of diabetes and PDAC association[16]. Importantly, other population based studies did not find any association between long-standing diabetes and the development of PDAC[17,18]. Thus, the elevated risk of PC in long-standing T2DM is confounded by the factor that may originate from a common soil of obesity and IR. Further, the role of different anti-diabetic medications as a risk modifier cannot be ignored while assessing the risk.

The Mendelian randomization (MR) studies looking into causal association between long-standing diabetes and PC have yielded conflicting results. While some studies showed causal association, others did not[19,20]. A pooled analysis performed on MR studies including 8374 PC patients by Yuan *et al*[21], found an odds ratio (OR) of 1.08 (95%CI: 1.02-1.14; P = 0.009) for this association. Although this evidence suggests a modest increase in the risk of PDAC in long-standing T2DM, more studies are required to confirm this association in future.

RISK OF PC IN TYPE 3C DIABETES

Chronic pancreatitis is defined as the chronic progressive inflammation and fibrosis of the pancreas caused by various aetiology and finally results in both endocrine and exocrine pancreatic dysfunction[22]. Diabetes is found in 35%-50% of subjects with CP in the observational studies[23-25] and the prevalence of DM increases with the increasing duration of CP and may reach up to 90%[25]. This type of diabetes is known as type 3c diabetes. Diabetes is more common in patients with pancreatic calcifications, pancreatic exocrine insufficiency and those who underwent surgery[23,24]. In a meta-analysis including fifteen studies (8970 patients), the incidence of DM was 30% and the prevalence increased after 5 years of CP diagnosis[26]. Diabetes in CP is often difficult to manage as a significant proportion of subjects require insulin therapy[23]. Importantly, CP itself is a risk factor for the development of PC. Kirkegård *et al*[27] had shown the risk of PC in CP varies with the duration of the disease and the effect estimates were 16.16, 7.90 and 3.53 at 2, 5 and 9 years after the diagnosis of CP, respectively. Another important entity is fibro-calculous pancreatic diabetes (FCPD), also known as tropical calcific pancreatitis, a relatively common cause of type 3c diabetes in certain tropical countries. FCPD also carries a very high risk for the development of PDAC[28].

Thus, it is important to look for CP in a given patient of diabetes and a closer follow-up with appropriate imaging is needed for diagnosis of PC in suspected cases. Since CP patients are often malnourished, progressive weight loss or anorexia despite adequate glycaemic control should alert the clinician for the possibility of PDAC.

RISK OF PC IN NEW-ONSET DIABETES

NOD has been considered as an important metabolic marker for the development of PDAC within the first 2-3 years of its diagnosis. NOD serves as a harbinger of PDAC in patients more than 45-50 years of age and hence calls for a careful follow up[29-31]. An earlier study demonstrated a 0.85% chance of development of PC within 3 years of diagnosis of diabetes in persons aged 50 years or more[32]. This study also showed that the risk was almost 8 times higher in patients with NOD. In a large cohort of 2.3 million Israeli population, a very high risk for developing PC was observed both in women and men (HR of 15.24 and 13.88 respectively) during the first year after the diagnosis of diabetes[33]. Two meta-analyses[13,14] also showed a 5-7 times elevated risk of PDAC in NOD, particularly within first year of diagnosis. Such an association was confirmed in different ethnicities like African Americans, Latinos[34] and Asians [35].

Agarwal *et al*[36] reported a very high prevalence of DM (68 %) in patients with PC compared to age matched other cancers subjects or non-cancer controls. Similarly, the number of NOD within the preceding 36 mo was markedly higher in PC than the other two groups (40% *vs* 3.3% *vs* 5.7%). About 50%-74% of the PC related diabetes is of recent onset (< 2-3 years duration)[6,37]. The prevalence of dysglycaemia in PDAC was more when standard oral glucose tolerance test (OGTT) was used instead of fasting glucose levels for diagnosis (78% *vs* 45%)[36,38]. The abnormalities in glucose metabolism are frequently missed in PDAC. The importance of making a preoperative diagnosis of glucose abnormality needs to be emphasized in this setting as it is shown to influence the surgical policy in up to 15% of patients[39].

It was also observed that a significant proportion of NOD in patients with PDAC resolved after pancreatic resection[37]. This indicates that PDAC by itself is causally related to the development of NOD, which is an early and specific biomarker for PDAC rather than a mere consequence. Besides the NOD, a deterioration of the existing glycaemic control in the form of elevated glycated hemoglobin **(**HbA1c) has also been associated with the development of PDAC[38].

MECHANISM OF DEVELOPMENT OF PC IN LONG-STANDING T2DM

The potential mechanism responsible for the development of PDAC in long-standing diabetes is poorly understood (Figure 1). The proposed theories are: (1) IR and the resulting direct effect of hyperinsulinemia[40]. A very recent study performed in a large prospective cohort (> 0.5 million subjects with a median follow-up of 8.4 years) has shown that higher IR as assessed by homeostatic model assessment- IR (HOMA-IR) is an important and independent risk factor for PC related mortality even in patients without diabetes[41]; (2) Cancer promoting role of the IGFs[42]; (3) The potential role of hyperglycaemia itself to alter several biochemical pathways involved in the carcinogenesis; (4) The synergistic effect of obesity and inflammation ('the common soil hypothesis'); and finally (5) Genetic predisposition to both these conditions. Experimental evidence is emerging to explain the molecular mechanism linking T2DM and PDAC. They include the roles of cellular senescence promoted by both T2DM and obesity[43], advanced glycation end products and its receptor[44], metabolic reprogramming by hyperglycaemia[45] and the interplay between non-alcoholic fatty pancreas development in the milieu of obesity and diabetes[46].

MECHANISM OF DEVELOPMENT OF NOD IN PC

PDAC by itself induces a potential 'diabetogenic' state. PC-associated NOD is grouped under Type 3c diabetes, which also includes diabetes caused by CP[47]. The mechanisms linking PC and NOD are shown in Figure 2. The hypothesis that NOD is the result of destruction of the endocrine pancreas by PDAC is not a plausible explanation because NOD can be present even before PC becomes radiologically detectable[48] and has also been shown to improve after surgery[37]. Hence, it is essential to search for systemic mediators of NOD in PC; until now, only a few of them have been substantiated.

Role of insulin resistance

Initial pioneering studies have shown that PC-associated NOD causes marked impairment in insulin action[49-51], more profound in patients who had diabetes[50].

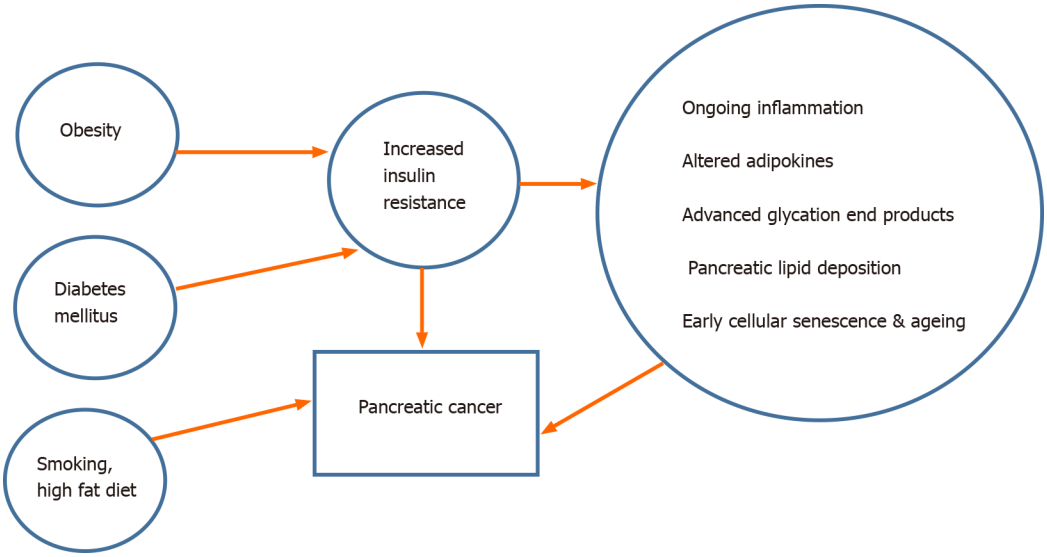


Figure 1 Schematic diagram depicting the interplay of various proposed factors leading to development of pancreatic cancer in long-standing diabetes mellitus.

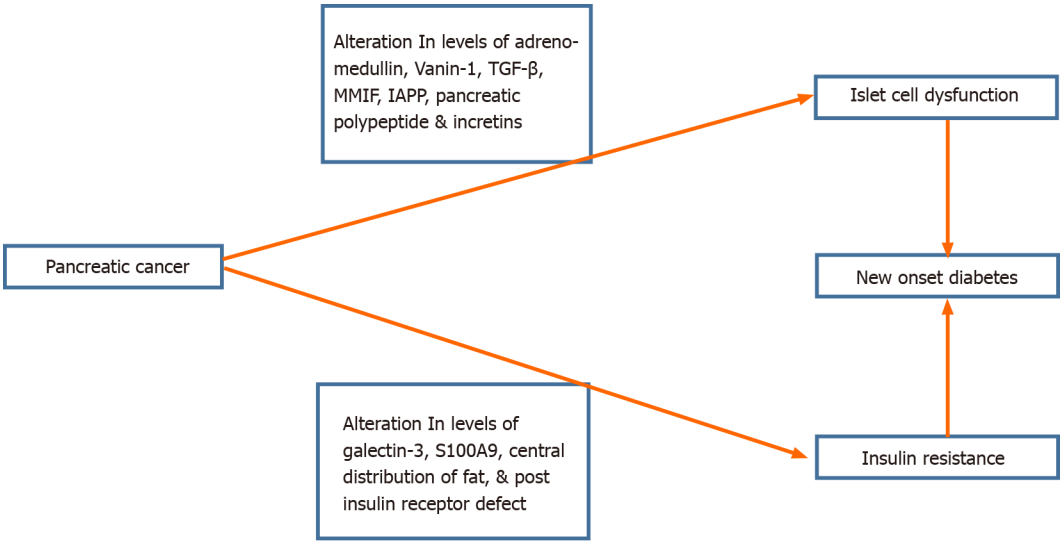


Figure 2 Schematic diagram for the possible mechanism for the pathogenesis of new-onset diabetes mellitus in pancreatic cancer. IAPP: Islet amyloid polypeptide; MMIF: Macrophage migratory inhibiting factor; TGF-β: Transforming growth factor-beta.

Insulin mediated glucose entry at the level of skeletal muscle was particularly found to be impaired significantly[52]. Interestingly, Permert *et al*[49] also demonstrated an improvement in whole body insulin sensitivity following surgery by using a hyperglycaemic clamp, which is considered to be the gold standard in evaluation of insulin sensitivity.

Thus, IR is an important determinant of the PDAC-related NOD, but the underlying mechanisms remain to be further studied. Currently available studies have suggested that IR may be related to the post-insulin receptor defect, particularly involving glycogen synthesis and storage pathways[53]. Recent experimental studies have found that PDAC-associated exosomes can inhibit the insulin receptor signalling pathway downstream of the receptor causing IR in skeletal muscle[54]. Another proteomic study revealed that galectin-3 and S100A9, which are overexpressed in PDAC-related NOD, can induce IR and can also serve as markers in distinguishing this entity from T2DM[55].

The role of islet amyloid polypeptide (IAPP) in the development of IR was initially suspected but later its clinical utility was not proven[56]. It was also suggested that PDAC-related NOD is due to the differential effect of ectopic fat as PDAC is characterized by subcutaneous fat loss and preservation of visceral fat[57]. However, a recent

study found that 30-18 mo before the diagnosis of PDAC, a significant proportion of patients had developed hyperglycaemia without any discernible change in the muscle or fat compartments[58,59].

Role of islet cell dysfunction

Pancreatic islet cell dysfunction is likely to be a crucial factor in the development of PDAC-related NOD. The morphology of the pancreatic islet in PC was recently characterized by Nagpal *et al*[60]. They demonstrated a significant reduction in islet density, beta and alpha cell area in PC compared to T2DM/control subjects. PC-related DM had lower IAPP deposition than T2DM. The lower IAPP deposit in PC related DM was also noted in an earlier study[61]. Future studies should explore the functional impact of such morphological changes.

Earlier studies have demonstrated a lower C-peptide response to glucagon stimulation suggestive of a beta cell secretory dysfunction in PDAC[62]. Beta cell function as assessed by HOMA-B was also found to be lower in PDAC patients with higher fasting glucose and diabetes[63]. In experimental studies, it was shown that beta cell in PDAC secrete increased amount of amylin preferentially while insulin secretion is diminished[64-66].

There is an experimental evidence for inhibition of insulin secretory function of the beta cell by adrenomedullin, which is released from PC-associated exosomes[67]. The role of adrenomedullin inhibiting beta cell insulin secretion in response to glucose was previously shown[68]. Moreover, adrenomedullin upregulation was noted in PC and its role in IR was also demonstrated[68]. Adrenomedullin was found to be a mediator for the increase in the exosome-induced lipolysis of the subcutaneous fat in PDAC [69]. The role of adrenomedullin as a screening biomarker is currently investigated in a prospective cohort study to identify patients with NOD and underlying PDAC[70].

Vanin-1 helps in hydrolysis of pantetheine and synthesis of vitamin B5 and cysteamine, which are required for lipid, energy and coenzyme A metabolism[71]. The role of vanin-1 is implicated in PDAC-induced paraneoplastic islet cell dysfunction, predominantly mediated by decreasing glutathione and elevating oxidative stress[72]. The same group earlier identified vanin-1 as a distinct marker of PDAC-related DM based on gene expression profile of the peripheral blood[73]. The role of transforming growth factor-beta (TGF-β) in the destruction of pancreatic beta cell had also been shown in animal studies[74,75] The macrophage migratory inhibiting factor was overexpressed in PDAC and was shown to decrease the beta cell secretory function [76].

A recent study has demonstrated that the markers of beta cell de-differentiations are consistently higher in non-diabetic PDAC patients suggesting the possible role of beta cell reprogramming in the early beta cell dysfunction even before the appearance of hyperglycaemia[77]. This dedifferentiation might be potentiated by the inflammatory milieu triggered by the PDAC.

Studies relating to alpha-cell function with PDAC-related DM are lacking. One study showed a higher glucagon/insulin ratio as a marker of NOD in PDAC[78]. Another small study revealed hyperglucagonemia in PDAC-related DM patients[79]. However, further studies are required to delineate the role of alpha cell dysfunction in PDAC-related diabetes.

Pancreatic polypeptide (PP) is released from the PP cells predominantly located in the head of the pancreas. PP cells have an important paracrine action including suppressive effect on glucagon secretion from alpha cell. Interestingly, one study had reported diminished PP response at 30 min following a mixed meal challenge in PC-related DM patients as compared to T2DM[80]. This was seen in tumours located in the ventral part of the pancreas. However, another study did not find any difference in fasting PP levels between PDAC-related DM, CP and T2DM[81]. Further studies should explore the role of PP in NOD and its use as an effective screening tool for PDAC.

Very few studies have evaluated the role of incretin hormones in the pathogenesis of PDAC-related DM. Interestingly, one study reported a lower gastric inhibitory polypeptide (GIP) and PP secretion in PDAC patients with diabetes as compared with T2DM patients, without any difference in glucagon-like peptide 1 (GLP-1) response [82]. Importantly, those with NOD or prediabetes with weight loss (> 2 kg) had significantly lower GIP. However, further studies are required to confirm this association. In-vitro studies had demonstrated that a lower GIP and GLP-1 response might be related to the inhibitory effect of the PDAC-exosomes on the proprotein convertase subtilisin/kexin type 1/3 enzyme which is responsible for cleaving the pro-glucagon molecule to generate the incretin peptides[83]. This study suggested the possibility of pancreatic exosome mediated dysfunction of the incretin hormones in

the gut.

EARLY DETECTION AND/OR SCREENING MODELS FOR PC IN DM

Clinical indicators

Till now, the recommendations regarding systemic screening of a person with diabetes to identify PDAC are not standardized. But, whom to screen and how to screen is not defined clearly by any guidelines till now to the best of our knowledge. So, it is necessary to develop a screening tool based on NOD and other risk factors. Since the yield of screening in such population is low, whether systematic screening is cost-effective and practically feasible remains an area of active debate.

The screening of patients with diabetes for PC is based on filtering of diabetes patients based on presence of associated clinical factors or level of biomarkers or a combination of such factors. NOD within 3 years of diagnosis increases the risk of PDAC 6-8 times more than the general population, but the prevalence of PDAC in such circumstances is low (0.8%-1%)[32].

It is a challenge to differentiate PC induced NOD from the more commonly encountered T2DM based on clinical and biochemical factors in clinical practice (Table 1). There are many overlaps between these two entities[84-86]. Munigala *et al* [87], identified age ≥ 65 years, heavy smoking, non-obese status at diagnosis, history of CP or gallstones as different risk factors of PC in a prospective cohort of NOD. One study reported a 40% higher risk of PC in patients with dyslipidaemia, although the association with specific lipid parameter was not mentioned[88].

Two other important factors that may provide a clinical clue for PC associated NOD are weight loss and worsening of hyperglycaemia. A continued weight loss in the presence of NOD was observed in a greater number of PC patients (59% *vs* 30%) than T2DM[89,90]. The amount of weight loss was also more in PC patients (8.3 ± 8.3 kg *vs* 0.8 ± 4.8 kg). Mueller *et al*[91] showed that weight loss of more than 10% had an adjusted OR of 3.58 (95%CI: 2.31-5.54) for development of PC. The presence of weight loss of more than 15% was not only associated with an increased odds of PC in NOD [91] but also in patients with long-standing diabetes[92]. Olson and colleagues showed that NOD and severe weight loss often occurred together before the diagnosis of PC [90]. Chen *et al*[11] observed that in a subject with NOD, when weight loss was unintentional or occurred in an individual with body mass index (BMI) less than 25 kg/m^2, then it substantially increased the risk of PC. Hence, weight change should be actively sought in elderly diabetes and warrant further investigation for PC.

Sah *et al*[58] reported worsening of hyperglycaemia, in the 18 to 6 mo before the diagnosis of PDAC. Similarly, rapid elevation of both blood glucose and HbA1c was observed by Huang *et al*[93] in the months preceding the detection of PC. The worsening of hyperglycaemia more often required the use of insulin treatment[90]. Thus, rapid deterioration of glycaemic control should alert a physician to screen for PDAC.

Another important feature is the loss of muscle mass, which is also known as sarcopenia. Sah *et al*[58] observed loss of subcutaneous adipose tissue even 6 mo before PDAC diagnosis. It was suggested that the preferential loss of subcutaneous adipose depot with relative preservation of the visceral adipose tissue might explain the IR and the worsening of glycaemic status[57]. However, a recent study did not find any difference in the prevalence of cachexia, skeletal muscle loss or weight loss between PC patients with or without DM[59]. Overall, sarcopenia suggests advanced disease and often portend poor survival, but its relationship with diabetes development need to be assessed in future studies[94].

Screening models

Interestingly, another upcoming approach is the development of a predictive model based on easily available clinical features in NOD. This model can identify NOD patients to be screened for PC and thus improve the detection rate while significantly decreasing the cost[30]. Sharma *et al*[95] came up with a model known as the Enriching New-Onset Diabetes for Pancreatic Cancer (END-PAC). This is a risk prediction model based on three different factors: change in weight, change in blood glucose, and age at the onset of DM. A score of 3 or more identified 78% of patients (n = 7/9) with 85% specificity. In the initial model, a score of more than 3 predicted a significantly increased risk of PDAC (4.4-fold) and a low END-PAC score of less than 0 had a very low risk of development of PDAC. This model was further assessed in a retrospective cohort of NOD patients (n = 13947) and 2% of high risk population (62 out of 3038)

Table 1 Factors that can help in differentiating pancreatic cancer associated new onset diabetes from type 2 diabetes mellitus

Clinical indicators	Biochemical markers
Age > 65 yr	Carbohydrate antigen 19-9
Heavy smoker	Galectin 3
Low body mass index	S100A9
History of chronic pancreatitis or gall stone disease	Insulin like growth factor-1
Recent worsening of hyperglycemia in an elderly patient	Osteoprotegerin
Weight loss associated with diabetes onset	Pancreatic polypeptide
Loss of subcutaneous fat and muscle mass in imaging studies like dual energy X-ray absorptiometry or magnetic resonance imaging	Thrombospondins- 1
	Vanin 1
	Matrix metalloproteinase-9
	MicroRNAs

were diagnosed with PDAC within 3 years yielding a sensitivity and specificity of 63% and 78% respectively at the score level of 3 or higher[96]. The positive predictive value (PPV) and negative predictive value were 2.0% and 99.7%, respectively. Another model was proposed by Boursi *et al*[97] based on The Health Improvement Network, which is a large primary care electronic research database from the United Kingdom. This prediction model included several easily available clinical parameters like age, BMI, change in BMI, presence or absence of smoking, use of proton pump inhibitors and other anti-diabetic medication including metformin. The laboratory parameters include levels of hemoglobin, creatinine, and alkaline phosphatase, HbA1c and cholesterol. The area under the curve (AUC) for the final model was 0.82 (95%CI: 0.75–0.89) and at a risk threshold of 1% for screening for PDAC, around 6% of patient with NOD would have to undergo systemic screening. The sensitivity, specificity and PPV at this level were 44.7%, 94.0% and 2.6%, respectively. Thus, though these model systems are encouraging and can narrow down on the screening population, they are limited by poor sensitivity and lower PPV[30] and further improvement is required before routine clinical use. Recently, a protocol of a multicentric, prospective observational study (NODES Trial) has been published, which intends to follow up new-onset (≤ 6 mo) diabetes patients over 60 years of age with both clinical and valid biomarkers [98]. The study also aims to evaluate for biomarkers that can distinguish patients with PDAC more precisely. Such studies will be invaluable in understanding and defining a screening protocol in NOD patients to identify PDAC as early as possible.

BIOMARKERS IN THE SCREENING OF PC IN DM

The role of different biomarkers in assisting the early diagnosis of PC among DM patients is crucial. A plethora of studies on different biomarkers have been published in the literature, though till now, none of them have reached the routine clinical use. The search for an easy-to-use clinically useful and cost-effective marker is still ongoing. Finding of suitable biomarkers is a difficult task in a relatively uncommon disease like PC and moreover, presence of diabetes can confound the measurements of different biomarkers in such setting[30]. A detailed discussion on this topic is out of the purview of this article, but a brief description on the latest biomarkers are discussed here. The proposed biomarkers are either measured in the blood or tissue fluids and they are the result of the 'multi-omics' studies involving proteomics, genomics and metabolomics.

Hormones involved in glucose homeostasis

The biomarkers, which draws our attention first is the biomarkers related to the glucose metabolism. Sharma *et al*[99] have shown that rising fasting plasma glucose itself predates the development of PDAC (36-60 mo before PDAC diagnosis) and is often related to the size of the tumour. Though fasting blood glucose levels increased concordantly with the volume of the tumour, no such relationship with the tumour gradation was reported. Another study reported a higher serum glucagon/insulin

ratio with a cut-off of 7.4 ng/mIU could differentiate PC induced NOD from T2DM with 77% sensitivity and 69% specificity[78]. A study demonstrated a higher glucose stimulated glucagon in PC patients with DM, suggesting glucagon as a potential biomarker[79].

Incretins involved in glucose homeostasis

The role of different gut polypeptides involved in the glucose homeostasis was also studied in PDAC patients. It was found that a significantly lower plasma concentrations of GIP and PP in patients with PC irrespective of the degree of glucose intolerance as compared to the T2DM and normal healthy controls[82]. A diminished PP response to a mixed meal was also observed earlier in PDAC associated diabetes in a small study[80]. However, another study could not find a difference in fasting PP levels between PDAC patients with or without diabetes and T2DM[81]. Thus the blunted PP response in PDAC can serve as an important tool for screening for PDAC, but the time-line is not well established and studies with a larger sample size may further consolidate PP as an important biomarker.

Carbohydrate antigen 19-9

A study by Choe *et al*[100] has shown that in asymptomatic NOD patients, a higher carbohydrate antigen 19-9 (CA19-9) levels above the upper normal limit had a 5.5 times risk of developing PC within 2 years of diagnosis. Another retrospective analysis showed similar results and found that the odds for development of PC in NOD patients with elevated CA19-9 was consistently higher, particularly in patients with elevated bilirubin levels[101]. Murakami *et al*[102] proposed that a cut-off of serum CA19-9 level of 75 U/mL can discriminate between patients with diabetes with or without PC. At this cut off, the sensitivity and specificity of CA19-9 for PC was 69.5% and 98.2%, respectively, while the AUC was 0.875 (95%CI: 0.826-0.924). A combination of elevated CA19-9 and carcinoembryonic antigen was also shown to detect PC among DM patients[103]. However, it is important to note that the utility of CA19-9 may be limited by the fact that it is affected by the levels of glycemia. CA19-9 levels must be interpreted in the context of ongoing glycaemic control and patients with diabetes per se may have elevated CA19-9[104].Thus there is a need to optimize the CA19-9 cut off level in DM patients for PC detection.

Thrombospondin -1

Another promising biomarker is thrombospondin-1 (TSP-1), a multimeric protein with anti-angiogenic properties. TSP-1 levels were found to be lower in PDAC patients, particularly those with diabetes as compared to non-diabetes and this lower levels were detected even 24 mo before the diagnosis of PDAC[105]. According to this study, TSP-1 levels in combination with CA19-9 yielded an AUC 0.86 in the detection of PDAC. Importantly, a lower TSP-1 levels were also noted in PDAC associated diabetes but not in the long-standing T2DM.

Vanin-1 and matrix metalloproteinase 9

Vanin-1, a protein involved in the oxidative stress pathway was found to be associated with paraneoplastic islet cell dysfunction (see earlier) and can serve as a potential biomarker in detecting PC among DM patients. Huang *et al*[73] have shown that the levels of *Vanin-1* genes were significantly upregulated in PDAC and an elevated levels of both vanin-1 and matrix metalloproteinase 9 (MMP9) in serum using quantitative real-time polymerase chain reaction could differentiate PDAC associated diabetes from T2DM. The AUC for the combination of both Vanin-1 and MMP9 was 0.950 with a sensitivity of 95% but the specificity of 76%. A combination of CA19-9 and MMP9 was also found to be helpful in discriminating PDAC-related diabetes from T2DM with an AUC of 0.886[106].

Galectin-3 and S100A9

Galectin-3 is a β-galactoside–binding lectin involved in the proliferation, migration and invasion of PC cells[107] whereas S100A9 protein is involved in the inflammation through toll-like receptor-4[108]. Liao *et al*[55] have shown that levels of both galectin-3 and S100A9 were higher in PDAC related DM than T2DM. They also found that the serum levels of both galectin-3 and S100A9 proteins can differentiate between PDAC related DM and T2DM with the AUC of 0.83 (95%CI: 0.74–0.92) and 0.77 (95%CI: 0.67–0.87) respectively.

MicroRNAs

Alteration in the profile of serum microRNAs (miRNAs) have been postulated as important biomarkers in recent times. One study reported that a panel of six miRNAs (miR-483-5p, miR-19a, miR-29a, miR-20a, miR-24, miR-25) could differentiate between PDAC related DM and T2DM[109]. The AUC for the differentiation of these two entities was 0.885 (95%CI: 0.784-0.986). However, the micro RNA profiles (miR-192, miR-196, miR-200, miR-21, miR-30 and miR-423) were found to be similar in PC patients with or without DM in a study by Skrha *et al*[110]. This list of miRNAs will be increasing in the future but the potential ones should come out from the prospective follow-up studies of the NOD patients.

Metabolomics

Studies have used extensive metabolomics approach either through liquid or gas chromatography and mass spectrometry or nuclear magnetic resonance imaging technique to identify various metabolites in order to identify specific biomarkers that can differentiate between PDAC-related DM and T2DM. One study revealed a distinct signature of 62 different serum metabolomes in PC related DM as compared to T2DM [111]. Out of them, two metabolites namely N-Succinyl-L-diaminopimelic-acid and PE (18: 2) had shown good sensitivity (93.3%) and specificity (93.1%) for PC in the logistic regression analysis. A recent nuclear magnetic resonance based study also identified a panel of eight metabolites with good accuracy (more than 80%) in the discrimination of PC and long-standing T2DM patients[112]. In future, possibly a panel of metabolites will help us to improve the precision medicine in identifying the cases requiring close follow-up for detection of PC among diabetes patients.

Other new biomarkers

There have been several other biomarkers proposed for the differentiation of the PDAC associated diabetes from T2DM. A very recent analysis of several immune related proteins including cytokines, chemokines and adhesion molecules revealed that a panel of different molecules (GM-CSF, IL-31, RANTES, RESISTIN, FASL, & ICAM1) were different between PDAC related DM and T2DM with an AUC of 0.96 (0.93–1.00)[113]. This study paved a new way in the screening of PC in diabetes patients. The other tools that can be used to screen PC are plasma free amino acid index[114], combination of either neutrophil-to-lymphocyte ratio or platelet-to-lymphocyte ratio and CA19-9[115], angiopoietin-like protein 2[116] among others with reported variable AUC.

In summary, although a great numbers of promising biomarkers have been studied to detect PC early in diabetes patients, a very few have reached routine clinical use as of now. As more translational research is emerging, the main requirement of a panel of clinically useful biomarkers for early detection of PC in DM will be fulfilled in near future.

DIABETES AND THE TREATMENT OUTCOMES OF PC

Diabetes has an important role to play as a prognostic marker in PC patients. Pancreatectomy is the initial management strategy in PDAC. Currently, the evidence that diabetes may portend an unfavourable impact on the overall outcome of PC, particularly after surgery is not concrete[117]. Whether the treatment of the diabetes modifies this risk is also not clear at present. Hank *et al*[118] showed that diabetes subjects had a poor median overall survival (18 *vs* 34 mo; $P < 0.001$). Moreover, diabetes was associated with higher 30-d mortality (3.2% *vs* 0.8%; $P = 0.019$). Importantly, a larger tumour size, a greater number of lymph node involvement and more peri-neural invasion were seen in diabetes patients with PC. A negative association of diabetes with overall survival was also noted in a meta-analysis[119]. However there are studies which do not agree on such association[120,121], and rather showed paradoxical reduction in the risk of death[122]. A 2013 review showed that diabetes patients had a higher risk of post-operative complications (45% *vs* 35%)[123]. Baseline HbA1c more that 6.5%-7.0% was also found to be associated with a shorter survival[124,125].

Long-standing DM vs new onset DM

Although studies have shown a poor outcome in all diabetes patients, the relative role of duration of diabetes on PC outcome needs further clarification. Very few studies

have shown the stratified analysis based on diabetes duration. Long-standing diabetes was found to have an association with diminished survival in prospective studies [126]. This was also confirmed in a meta-analysis[127] involving 18 studies (16181 patients). Several other studies[117,128,129] did not find any significant effect of long-standing diabetes on the survival in PC. Jeon *et al*[117] reported impact of long-term diabetes on decreased survival in those with resectable PDAC (HR, 1.42; 95%CI: 1.13–1.78) but not in advanced disease suggesting a role of staging in the outcome. It is important to note that the association of diabetes with prognosis became non-significant in most of the studies, after adjusting confounders like age, gender, BMI, smoking status and staging of the disease[128]. The evidence that diabetes patients can have a relatively larger pancreatic tumour size is well established[118,121,130,131].

On the other hand, the evidence is more consistent for a poorer outcome associated with NOD. A 2017 meta-analysis showed that only NOD was associated with shorter survival but not long-standing DM[119]. Similarly, other studies found that only NOD was a significantly independent predictor of decreased survival[129,132]. Importantly, Lee *et al*[133] have shown that NOD carries a higher risk of recurrence after pancreatic resection and may be a factor responsible for the poorer outcome. In contrast Jeon *et al* [117] did not find any impact of NOD on survival. Another point to consider is that whether post-surgery improvement of NOD has any impact on outcome. Though a study reported increased survival in patients where diabetes was resolved following surgery[134], future studies should substantiate this finding.

Impact of DM on the outcome after chemotherapy

Studies assessing the impact of diabetes in PC patients receiving chemotherapy have shown that, a prior diabetes status might be associated with a higher risk of death. Kleef *et al*[130] demonstrated a higher mortality rate in diabetes patients receiving adjuvant chemotherapy [HR 1.19 (95%CI: 1.01-1.40)]. Similarly, Hank *et al*[118] showed that median overall survival was lower in diabetes patients who received neo-adjuvant chemotherapy as compared to non-DM patients (18 mo *vs* 54 mo; $P < 0.001$). Another study showed diabetes to further add to the poorer outcome in metastatic disease treated with gemcitabine[135]. A meta-analysis looking at the impact of diabetes on the outcome following chemotherapy in PC (1034 with DM and 3207 without DM) demonstrated a lower survival and higher risk of death after chemotherapy in DM patients[136]. A high preoperative HbA1c was also found to be associated with non-completion of adjuvant chemotherapy and a higher risk of metastasis[137]. Diabetes also affects the survival in very advanced PC patients receiving palliative chemotherapy[138].

The mechanism behind the poorer outcome in PC with diabetes is not certain. Diabetes is associated with larger tumour size and hence a higher tumour stage. Hyperglycaemia has been shown to hasten the tumour development *via* sterol regulatory element binding protein 1 pathway[139]. There is also suggestion for an alteration in the tumour microenvironment in the presence of an elevated blood glucose level. Indeed, experimental studies have shown that hyperglycaemia increases the metastatic ability of the PC through aggravated hypoxia[140] or by increasing the perineural invasion[141]. The role of glycaemic variability is also suggested as a risk factor for promoting local invasion and metastasis *via* the retinoic acid receptor beta-runt related transcription factor 3-type VI collagen alpha 1 chain pathway[142].

EFFECT OF PC TREATMENT ON DIABETES

There is a complex relationship existing between patients undergoing surgery for PC and their glycaemic status. Glycaemic control is expected to worsen following pancreatectomy considering a significant loss of beta cells. However this is not often observed in clinical practice, particularly in patients with NOD after surgery. Studies[143-146] have either shown a significant improvement in their glycaemic control (75%) or resolution of NOD (20%-65%) after pancreatic surgery. It has also been reported that resolution of preoperative NOD after pancreatectomy may be a sign of a favourable outcome[134]. NOD has also been described in 15%-20% of patients[143,145,146] after surgery. One study reported deterioration of the glycaemic control in up to 40% post-operatively[147],when formal tests like OGTT and *i.v.* glucagon stimulation test were used. In the meta-analysis by Beger *et al*[148], cumulative incidence of NOD was found to be 15.5% after pancreatico-duodenectomy for malignant pancreatic tumours. Hence, it is necessary to assess the glycaemic status after pancreatico-duodenectomy even in those with pre-operative normoglycemia to achieve a better metabolic control after the

surgery.

ANTI-DIABETIC MEDICATIONS AND PC

Since diabetic patients will be receiving several medications for controlling hyperglycaemia, it is important to consider their effects in the context of PC. There are many excellent reviews[149] already available in this regard and we highlight salient points based on the recent available evidence.

Metformin

Metformin has garnered a lot of interest in recent times due to its anti-cancer effect and PC is not an exception. Metformin is the first line drug of choice for treating T2DM. A plethora of studies have looked into the three key aspects of metformin and their role in PC. They are: (1) Metformin as a risk modifier of PC development in T2DM[150-152]; (2) Effect of metformin on the overall survival following therapy[153]; and (3) Metformin as an adjuvant therapy in diagnosed PC[154].

Studies regarding metformin treatment as a risk modifier of PC have yielded mixed results. While some studies have shown risk reduction of PC in metformin users[155], other studies did not find such a beneficial impact of metformin in PC risk reduction [156,157] and even reported an increased association risk in metformin users[151]. Though earlier pooled analysis[150] had shown a decreased risk of PC, the studies included in those analysis were mostly retrospective and met with the significant lead time bias. Another complicating issue regarding the risk estimation is that the long duration of diabetes already present is itself a potential risk for PC and the NOD heralding the onset of PC often complicates the scenario further. Therefore, recent studies with better statistical designs are warranted to establish the role of metformin in PC prevention in a concrete manner.

Animal studies have shown that metformin decreases the PC cell proliferation[158, 159], its invasiveness[160] and thereby reduces the metastatic potential of PDAC[161]. Studies have also shown that metformin has a sensitization effect on chemotherapy, particularly gemcitabine[161,162]. The inhibition of TGF-β pathway is one of the several underlying mechanisms proposed to explain these effects[75,160]. Considering this finding, metformin might be expected to have beneficial result in PC. However, the clinical studies performed to assess the benefits of metformin have shown conflicting results. While few studies have suggested the survival benefit of metformin in PC patients[163-165], two phase 2 randomized controlled trials (RCTs)[166,167], observational studies[168,169] and other meta-analysis[170] refuted such finding.

The benefits of metformin observed in some earlier studies may be attributed to the immortal time bias that is inherent to meta-analysis studies[171]. Indeed, a meta-analysis taking into the account of immortal time bias did not show any additional survival benefit of metformin in DM patients with PC[170]. According to this meta-analysis, the effect size of reduction in the risk of survival was exaggerated by 18%. Again, the null effect of metformin on survival shown by the two RCTs was also flawed by the fact that metformin treatment was started late in the disease course and metastasis has already happened, leaving a small room to assess for the effect of metformin on survival outcome[166,167]. For this reason, it is important to focus on this area with well-designed RCTs in an earlier stage, including both DM and non-DM population. Further evidence is required to recommend treatment with metformin in PDAC patients with concurrent DM. Mild hyperglycaemia with obesity in early stage PC patients may be an ideal indication to start metformin. In summary, the risk reduction of PC and the overall survival following metformin therapy are not observed in recent well designed studies with improved statistical analysis taking into consideration of immortal time bias. Future phase 3 RCTs will be helpful in this context, mainly in selected PC candidates[172].

Insulin and insulin secretagogue

Insulin has a definite role in the pathogenesis of PDAC as hyperinsulinemia and IR are important risk factors for the development of PDAC (see above). Whether clinical use of insulin has any impact on PDAC development is a contentious issue. Long-term insulin use was not found to be a risk factor of PDAC development[173]. On the other hand, short-term insulin user (< 3-5 years) was found to have an elevated risk of PDAC (OR 5.60, 95%CI: 3.75-8.35)[173]. Perhaps this data reflects the other way around. It is likely that the worsening of hyperglycaemia or the severe hyperglycaemia requiring insulin injection might reflect the onset of PDAC or effect of PDAC on the

glycaemic control. Whereas few meta-analysis have suggested an elevated risk of PC in insulin users[174] the same evidence was not found in other studies[175] and also with newer insulin like glargine insulin[176]. In terms of survival benefit, insulin use had no impact on survival as shown in recent studies[120,177].

Insulin secretagogues like the sulfonylureas (SU) are also implicated as a risk modifier of PC. There are only few studies that have specifically looked into the link between SU and PC. However, studies including nation-wide cohorts[178], and meta-analyses[175] had pointed that SU use is associated an elevated risk of PC (OR varies between 1.5-1.7). However, with newer generation SU data is sparse and this association is further complicated by the effect of concurrent obesity and IR on the development of PC. Moreover, earlier analyses are met with significant methodological flaws and heterogeneity among studies[179].

Incretin based therapies: DPP-4 inhibitors and GLP-1 receptor agonist

Incretins are hormones secreted from the intestine and have a significant impact on the glycaemic control. GLP-1 analogues and inhibitors of dipeptidyl peptidase-4 (DPP-4) enzyme are established therapies for T2DM in clinical practice. Although there was a concern of acute pancreatitis and PC associated with their use from the initial preclinical[180] and adverse database review[181] studies, data regarding the risk for PC was inconsistent. Hence, both United States Food and Drug Administration and European Medicines Agency advised on continuous follow-up of patients started on these therapies for these two adverse events[182].

Earlier meta-analysis also did not find an increased risk of PC with DPP-4i treatment group[183]. Moreover, the recent meta-analysis involving 157 trials reporting PC (66897 patients in DPP-4 inhibitors and 61597 patients in control group) showed no associated risk with DPP-4 inhibitors use (OR: 0.84 [95%CI: 0.69-1.03], I^2 [for heterogeneity] = 0%). This association was found across different types of DPP-4i molecules and thus possibly reflects a class effect. Data from large population based studies also showed similar reassuring findings[184]. Due to several limitations of the trials like a shorter follow-up, reporting bias, small number of PC cases, it is important to keep a watch over this association in future. Moreover, one meta-analysis of the large cardiovascular outcome trials on DPP-4i showed an 75% increased risk of pancreatitis[185]. Such findings warrant longer duration follow-up studies and continued vigilance. A study[186] has shown that DPP-4i may be associated with increased risk of pancreatitis and PC in short-term without any relationship with exposure duration, thus implying that it might be the result of reverse causality rather than the DPP-4i exposure itself.

Similarly, more data are now available for different GLP-1 analogues. The larger cardiovascular outcome trials did not find any elevated risk of PC in GLP-1 analogue users[187,188]. Consequently, an updated pooled analyses from the cardiovascular outcome trials also did not show an excess risk of PC or pancreatitis with use of GLP-1 analogues[185]. However, it is noteworthy that such trials are not primarily meant to detect any increased malignancy risk. Thus, although the data is reassuring, a continued vigilance is warranted.

Other drugs (thiazolidinediones and sodium-glucose co-transporter type 2 inhibitors)

The other antidiabetic drugs are thiazolidinediones (TZD) and sodium-glucose co-transporter type 2 (SGLT-2) inhibitors. TZDs like pioglitazone and rosiglitazone primarily act through activation of the peroxisome proliferator-activated receptor-gamma pathway. This activation has direct and indirect implications in the PC biology. TZDs have shown to have inhibiting effect on several aspects of PC including cell proliferation and metastasis[189-191]. It also has the potential to modify the risk of PC through insulin sensitization, modification of the obesity and the inflammation [192]. However, these promising experimental findings of benefits of TZD have been replicated in clinical studies with mixed results.

While two meta-analyses did not find any association between TZD use and the risk of PC[175,193], one population based study had shown a protective role of TZDs against PC[178]. On the other hand, Lewis *et al*[194] demonstrated that TZD use might be associated with an increased risk of PC. As far as the prognostic role is considered, TZDs did not have any effect on survival[195,196].

SGLT2-inhibitors are the newest class of oral antidiabetic medication and have already made its place in the therapeutic algorithm of diabetes, owing to its cardiovascular benefits. Functional SGLT-2 are detectable in PC cells and hence, it was hypothesized that SGLT-2 inhibitors can inhibit tumour growth by blocking the entry

of the glucose within the cell[197]. An experimental study has shown canagliflozin, a SGLT-2 inhibitor to inhibit PC growth[198]. However, clinical studies are yet to confirm its effect on PDAC survival.

CONCLUSION

In this review, we have summarized the intricate relationship between DM and PC. Long-standing diabetes is considered as a risk factor for development of PC. On the other side, NOD in an elderly patient can be a manifestation of underlying PC. Though the exact mechanism remains to be eluded in future studies, the mechanism of the development of NOD in PC involves both IR and islet cell dysfunction. Diabetes has also been suggested to have an unfavourable effect on the overall survival of patients with PC.

Early detection of PC in a patient with DM is of utmost important and is a clinically challenging task. PC has a low prevalence in both general population and diabetes subjects. Thus, devising a strategy to screen diabetes population for PC is the need of the hour. There is an urgent need for a clinically useful and cost-effective screening tool to detect PC among patients with long-standing diabetes. The epiphenomenon of NOD can subserve as a potential clue along with recent onset worsening of glycaemic control and a continued weight loss. Apart from clinical pointers, many biomarkers have also been found to differentiate PC related DM from the commoner T2DM. Moreover, different clinical and biochemical parameters have been combined to develop different screening tools. Proper screening and early recognition of PC can improve the outcome of this devastating neoplasm.

Can we delay the occurrence or halt the progression of PC in a patient of DM? The strategies to improve IR like regular physical exercises, intermittent fasting, or low-fat diet can be explored in future. Moreover, other healthy behaviours like smoking cessation should be implemented in patients with long-standing DM. The role of glucose lowering medications like metformin in delaying the occurrence of PC needs to be explored further in longitudinal studies.

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REVIEW

Stress-activated kinases as therapeutic targets in pancreatic cancer

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Abstract

Pancreatic cancer is a dismal disease with high incidence and poor survival rates. With the aim to improve overall survival of pancreatic cancer patients, new therapeutic approaches are urgently needed. Protein kinases are key regulatory players in basically all stages of development, maintaining physiologic functions but also being involved in pathogenic processes. c-Jun N-terminal kinases (JNK) and p38 kinases, representatives of the mitogen-activated protein kinases, as well as the casein kinase 1 (CK1) family of protein kinases are important mediators of adequate response to cellular stress following inflammatory and metabolic stressors, DNA damage, and others. In their physiologic roles, they are responsible for the regulation of cell cycle progression, cell proliferation and differentiation, and apoptosis. Dysregulation of the underlying pathways consequently has been identified in various cancer types, including pancreatic cancer. Pharmacological targeting of those pathways has been the field of interest for several years. While success in earlier studies was limited due to lacking specificity and off-target effects, more recent improvements in small molecule inhibitor design against stress-activated protein kinases and their use in combination therapies have shown promising *in vitro* results. Consequently, targeting of JNK, p38, and CK1 protein kinase family members may actually be of particular interest in the field of precision medicine in patients with highly deregulated kinase pathways related to these kinases. However, further studies are warranted, especially involving *in vivo* investigation and clinical trials, in order to advance inhibition of stress-activated kinases to the field of translational medicine.

Key Words: Pancreatic cancer; Stress-activated protein kinases; Mitogen-activated protein kinases; c-Jun N-terminal kinases; Casein kinase 1; Small molecule inhibitor

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Core Tip: Since pancreatic cancer patients are generally confronted with poor prognosis, optimized therapeutic strategies are urgently needed. To establish new treatment options, efforts in drug development have increasingly focused on targeting protein kinases. In the cellular response to various stress signals, c-Jun N-terminal kinases (JNK) and p38 kinases as well as members of the casein kinase 1 (CK1) family are of special interest. Concentrating on pancreatic carcinoma in this review, we summarize the key roles of JNK, p38, and CK1 and provide an overview of recent achievements in the development of small molecule kinase inhibitors against these kinases.

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INTRODUCTION

Pancreatic cancer is a severe disease, with overall 5-year survival rates less than 10% and only very little improvement over the last decades[1]. It is currently the fourth most common cause of cancer-related deaths, and it is expected by 2030 that pancreatic cancer will have surpassed colon and breast/prostate cancer to move up to second rank of cancer-related deaths[2]. Contributing to the immense challenge of treating pancreatic cancer, dysregulation of multiple signaling pathways can frequently be detected. With the genetic hallmark mutation of *KRAS* in over 90% of all pancreatic cancer patients, the high relevance of kinase-driven pathways is underlined[3].

So far, classic chemotherapeutic agents have only shown moderate success in prolonging overall survival of patients suffering from pancreatic ductal adenocarcinoma (PDAC). However, more and more personalized therapy concepts are becoming promising options, especially the use of small molecule inhibitors specifically targeting newly identified drug targets, such as deregulated protein kinases. Of special interest are kinases activated in cellular stress situations, like mitogen-activated protein kinases (MAPKs) and members of the casein kinase 1 (CK1) family, which phosphorylate signal integration molecules like p53 and β-catenin finally resulting in activation of processes leading to cell cycle arrest or apoptosis.

MAPKs

MAPKs are key players in transducing extracellular stimuli into intracellular signaling cascades and therefore represent interesting drug targets. Multiple isoforms have been identified, which can be clustered into six groups of MAPKs. The most prominent of those are the extracellular-regulated kinases 1 and 2 (ERK1/2), the c-Jun N-terminal kinases 1, 2, and 3 (JNK1/2/3), and the p38 kinases α, β, γ, and δ[4,5]. As a response to various stimuli such as growth factors, cytokines, and environmental stress, MAPK-triggered phosphorylation of their target transcription factors (TFs) marks the endpoint of an intracellular kinase cascade. This cascade consists of ligands binding to their cell membrane receptors, recruitment of GTPase (*e.g.*, RAS) to the plasma membrane, and activation of MAPK kinase kinases (MKKKs or MAPKKKs, *e.g.*, RAF) as well as MAPK kinases (MKKs or MAPKKs, *e.g.*, MEK1/2)[5-7]. ERK1/2 belong to the best-studied kinases among MAPKs. Their relevance for pancreatic cancer has been well documented, especially as ERKs exert their functions downstream of mutant KRAS[8,9]. JNK and p38 can be grouped together as stress-activated protein kinases (SAPKs), as their pathways are regularly activated by environmental stressors, like nutrient deprivation, inflammatory cytokines, or ultraviolet irradiation[5,10].

CK1

A remarkable association with tumorigenesis and tumor progression has also been demonstrated for the CK1 family of protein kinases. Being among the first kinases described in history, involvement of CK1 isoforms in several essential signal transduction pathways has been reported within the last decades. As a key cascade in developmental processes, the (canonical) Wnt/β-catenin signaling pathway can also be involved in promoting cell proliferation through activation of oncogenes like c-myc

and cyclin D1[11,12]. All human CK1 isoforms were identified to fulfill negative as well as positive regulatory functions in canonical Wnt signaling, thereby either acting as tumor suppressors or contributing to Wnt-induced oncogenic processes[13-15]. CK1δ and ε might furthermore promote canonical instead of non-canonical Wnt signaling, consequently resulting in reduced JNK-mediated Wnt signaling and apoptosis[16,17]. In addition, apoptosis mediated by Fas can also be down-regulated by CK1δ- and ε-mediated stabilization of Bid[18]. Apart from signaling associated with proliferation, differentiation, and apoptosis, CK1 is also involved in further mechanisms of the cellular stress response, including functions in immune response and inflammation, regulation of microtubule dynamic processes, autophagy, and DNA damage-related signal transduction[19-21]. Especially well documented is the regulatory function of CK1 isoforms in p53-mediated signal transduction with CK1δ even forming an autoregulatory feedback loop with p53[19].

Cancer itself already forms a stressful environment on the tumor cells, induced by hypoxia and nutrient deprivation as well as metabolic and replication stress. Additionally, cancer cells face genotoxic stress exerted by chemo- and radiotherapies. In this regard, pancreatic cancer is no exception since tumors of the pancreas are known for their dense stroma with impaired vasculature and the association with metabolic stressors, like diabetic conditions. This review aims to elucidate the role of the stress-activated kinases: JNK and p38 but also CK1 in the pathogenesis of pancreatic cancer and their potential as therapeutic targets.

SAPKS: JNK AND P38

The first description of JNK as a 54 kDa protein kinase activated upon peritoneal cycloheximide injection into rats dates back to 1990[22]. So far, three different JNK isoforms have been identified in the human genome, including JNK1 (MAPK8), JNK2 (MAPK9), and JNK3 (MAPK10), whereas expression of JNK3 is mainly restricted to brain, heart, and testes[23]. Alternative splicing results in generation of at least 10 different JNK isoforms with molecular weights ranging from 46 kDa to 55 kDa[24]. Activation of JNKs is dependent on phosphorylation of threonine and tyrosine residues by upstream kinases. For JNK1, phosphorylation of Thr^{180} and Tyr^{182} within its kinase subdomain VIII has been demonstrated to be essential[25]. In the activation process of JNK1, the upstream kinases MKK4 and MKK7 both fulfill non-redundant functions, with MKK4 preferably phosphorylating Tyr residues while MKK7 favors Thr residues. Phosphorylation of Thr^{180} is sufficient for JNK1 activation, however, dual phosphorylation by both kinases is required for full JNK activation[26,27]. Further upstream of the signaling cascade, a large variety of at least 14 MKKKs can activate MKK4 and MKK7[23,28].

Merging the influence of multiple upstream kinases into fewer effector kinases enables the cells to respond to a variety of stimuli, like growth factors and cytokines, reactive oxygen species, physical interactions with other cells and the extracellular matrix, as well as cellular stressors[29]. The variety of different stimuli requires multiple cell membrane receptors engaging into the JNK pathway. These include G-protein coupled receptors, Wnt-receptors, transforming growth factor-β receptors, and tumor necrosis factor-α (TNF-α) receptors[30]. Signal transfer within the SAPK pathway is generally orchestrated by docking motifs for upstream kinases and downstream substrates, as well as scaffold proteins. Those scaffold proteins express docking sites for MKKKs, MKKs, and MAPKs and play an important role in the correct stimulus response through the kinase cascade[29,31].

Even more diverse than the upstream mediators are the possible JNK substrates. As all MAPKs, JNK is a proline-targeted serine/threonine kinase, thus preferably phosphorylating Ser-Pro as well as Thr-Pro motifs[4,32]. So far, the list of JNK substrates includes more than 100 targets, among them TFs like c-Jun, p53, c-myc, and β-catenin, microtubule-associated proteins, components of focal-adhesion-complex and cell-to-cell-adhesion, as well as apoptosis-regulating proteins like Bcl-2 and Bax [30]. Figure 1 shows an overview of MAPK-related cellular functions.

The first p38 MAPK was discovered in 1994, and today four isoforms (p38α, β, γ, and δ, corresponding to MAPK14, 11, 12 and 13) are known[33,34]. While p38α is ubiquitously expressed, the other isoforms show differential tissue distribution, with p38β being mainly expressed in the brain, p38γ in skeletal muscle, and p38δ in endocrine glands[35]. Dual phosphorylation of Thr^{180} and Tyr^{182} in a Thr-Gly-Tyr motif are required for full p38 kinase activation[36]. MKK3 and MKK6 specifically activate p38, but while MKK6 can activate all isoforms, MKK3 is unable to phosphorylate p38β

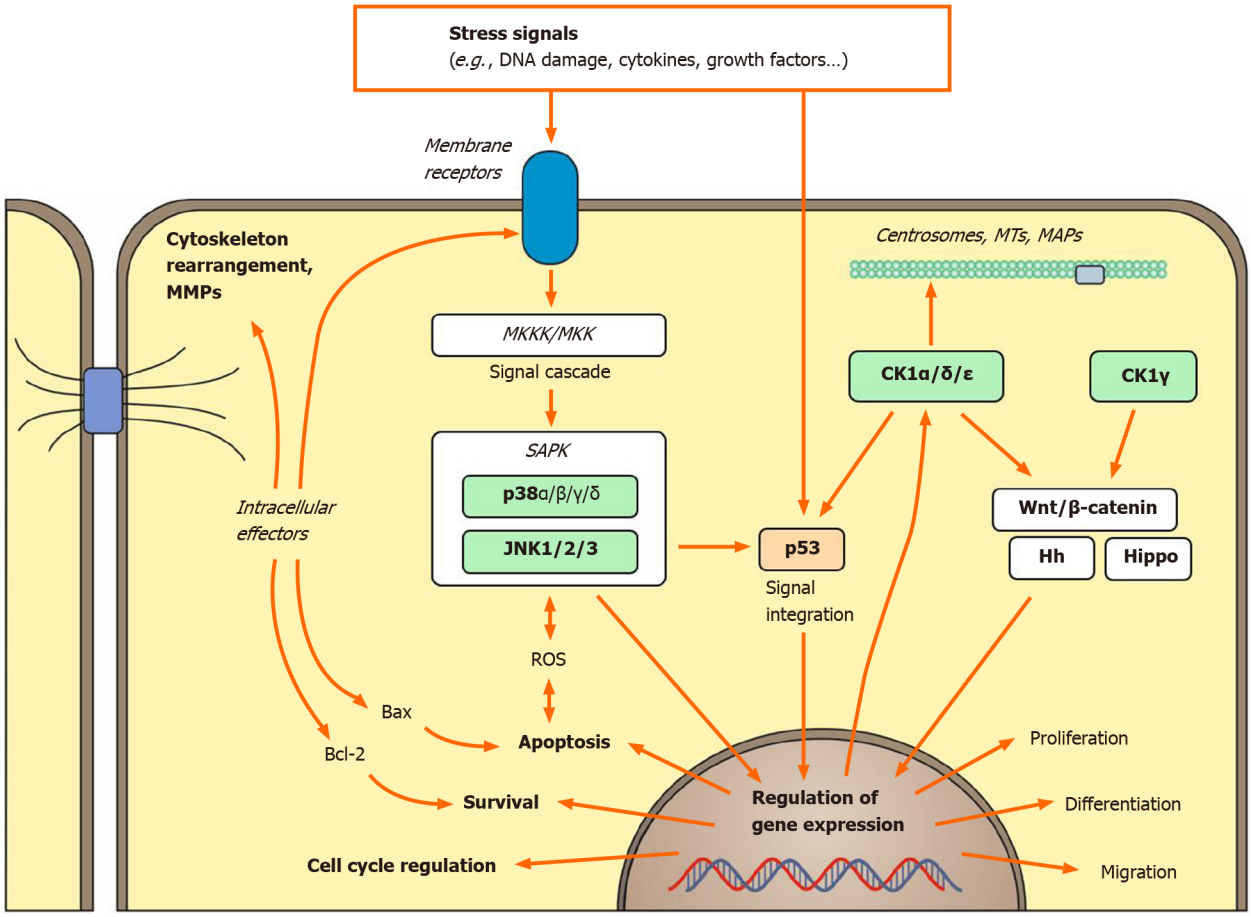


Figure 1 Signal integration mediated by stress-activated protein kinases. The stress-activated protein kinases (SAPKs) c-Jun N-terminal kinase (JNK) and p38 as well as the protein kinases of the casein kinase 1 (CK1) family are activated in response to endogenous and exogenous stress stimuli. Therefore, JNK and p38 are either activated directly or through upstream signaling cascades [mitogen-associated protein kinase kinase kinases (MKKKs), mitogen-associated protein kinase kinases (MKKs)] and subsequently exercise their functions through intracellular signal integrating effectors such as the transcription factors JNK, p53, c-myc, or β-catenin. In response to certain stress stimuli, the kinases of the CK1 family also take key functions in regulatory effects mediated *via* p53 or through various signal transduction pathways like the Wnt/β-catenin, Hedgehog (Hh), or Hippo pathway. Finally, cellular stress response including regulation of proliferation, differentiation, migration, cell cycle progression, survival, and apoptosis is initiated by modulation of gene expression. MAP: Microtubule-associated protein; MMP: matrix metalloproteinase; MT: Microtubule; ROS: Reactive oxygen species.

[37,38]. MKK4 can also contribute to p38 activation[39,40]. Multiple MKKKs contribute to the activation of MKK3 and MKK6, among which some are shared with the JNK pathway. By engaging specific MKKKs in response to defined stimuli, cells are enabled to elicit the correct stress response[35]. In T cells, a kinase cascade-independent pathway of p38α activation has also been described[41]. Similar to JNK, there are many p38-specific substrates, ranging from TFs to other protein kinases and apoptosis-regulating proteins[35].

Relevant SAPK-related pathways in cancer

The involvement of JNK and p38 in the pathogenesis of cancer has been studied extensively. Their role has been debated controversially since both can exhibit pro- as well as anti-tumorigenic functions[42,43]. For both kinases, the cellular effects provoked by JNK and p38 depend on the type, strength, and duration of the stimulus [44,45]. However, the influence of SAPKs on cancer development and progression is apparent as major cancer characteristics like cell proliferation, migration, and apoptosis are influenced by p38 and JNK. Pathways associated with these characteristics will now be discussed in general and specifically in pancreatic cancer.

A primary example of differential regulation in SAPK-related pathways is the interaction of JNKs and c-Jun. In non-stimulated cells, JNK2 activity leads to degradation of c-Jun, while after stimulation, JNK1 phosphorylates and stabilizes c-Jun. Consequently, knockdown of JNK1 decreased fibroblast proliferation through reduced activity of Activator protein-1 (AP-1), a TF of which phospho-c-Jun is a vital component[46,47]. Phosphorylation of c-Jun has also been identified as a critical step in RAS-induced tumorigenesis. Oncogenic RAS uses the same phosphorylation sites as

JNK on c-Jun and promotes transformation of rat embryonic fibroblasts, while *c-Jun*^{null} cells are resistant to RAS-induced transformation[48,49]. However, the role of JNK in this process is under dispute. In *TP53*$^{-/-}$ mouse embryonic fibroblasts, dual knockout of JNK1 and 2 reduced *KRAS*^{G12D}-induced transformation and suppressed *in vivo* growth. Furthermore, *KRAS*^{G12D}-induced lung tumor formation in mice was similarly reduced by JNK knockout[50]. This effect was in part attributed to JNK2, as only JNK2- but not JNK1-deprived mouse embryonic fibroblasts resisted RAS-induced transformation, although increased levels of AP-1 and phospho-c-Jun were observed[51]. However, not all transforming effects of RAS seem to be controlled by JNK, and in contrast to the before-mentioned studies, loss of JNK in RAS mutant cells may also contribute to enhanced tumorigenesis through apoptosis regulation[52]. A possible explanation for these findings is JNK-controlled cell cycle progression, as fibroblasts with knockdown of JNK2 show faster G1/S progression, while their JNK1-deprived counterparts show an opposing phenotype[46].

Besides proliferation, pro- and anti-apoptotic signals are also mediated by JNK in dependency of the stimulus. JNK regulates the expression of Bcl-2 family members and thereby influences apoptosis mediated *via* the mitochondrial pathway[44]. In TNF-mediated apoptosis, at early time-points, JNK activation triggers pro-survival pathways, while functioning JNK signaling is required for TNF-mediated apoptosis under persistent stimulation[53]. In murine cancer models, JNK1 was shown to promote chemically induced liver cancer, a finding that was also confirmed in human hepatocellular carcinoma[54,55]. On the other hand, knockdown of JNK1 rendered mice more susceptible to chemically induced skin tumors, while knockdown of JNK2 exerted an opposite effect[56].

The influence of p38 on oncogenesis is generally thought to be tumor suppressive, but protumorigenic functions like promotion of invasiveness has also been reported [44]. p38α controls proliferation through regulation of cell cycle progression at the G1/S and G2/M phases[57]. p38 can inhibit G1/S progression, *e.g.*, through downregulation of cyclin D1[40,58] or by phosphorylation of p53 and retinoblastoma protein [59-61]. Alternatively, p38 can also promote cell cycle progression through the induction of cyclin A or interference with the retinoblastoma protein pathway[62,63]. p38-mediated phosphorylation of p53 also activates the G2/M checkpoint in response to DNA double-strand breaks. Ironically, this also offers a survival benefit for tumor cells and increases therapy resistance, as DNA damaging drugs become less effective with functional p38 signaling[64-66]. Other tumor-promoting roles include formation of a pro-invasive phenotype through induction of matrix metalloproteinases and tumor cell dormancy, enabling metastatic relapse[42].

p38-related implication for involvement of the SAPK pathway in pancreatic cancer

The influence of the SAPK pathway on pancreatic cancer has been studied on patient samples as well as genetically engineered cell lines and mouse models, while patient cohorts are especially helpful to study population risk factors. Handra-Luca *et al*[67] offered an immunohistochemical analysis of the MAPK pathway in 99 surgically resected pancreatic cancer specimens. While high immunoreactivity for ERK1/2 was consistently associated with a worse prognosis, high expression levels of p38 could be associated with shorter recurrence-free survival in patients without adjuvant treatment. Strong staining for MKK4 was associated with increased proliferation[67]. Contrarily hereto, phosphorylated p38 with activated downstream TFs was identified as a favorable biomarker after surgical resection and associated with reduced number of lymph node metastases. Labeling of phospho-p38 showed no changes through the different cancer stages. Furthermore, pharmacologically inhibiting p38 *in vitro* and *in vivo* resulted in enhanced JNK signaling and enhanced cell growth[68]. Phospho-p38 was reported to increase during tumor progression, which is consistent with reports claiming that p38 has tumor suppressive functions during early carcinogenesis but switches towards a tumor promoting phenotype later on[69]. In our own previous work, we were able to dissect the isoform-specific functions of p38 in pancreatic cancer by genetically targeting p38 isoforms α and β. We confirmed an *in vivo* tumor suppressive phenotype of p38α but also showed a pro-invasive function. Additionally, we showed a tumor suppressive role of p38β, opposing p38α[70]. Interestingly, oncogenic KRAS induced activation of p38 and phenotypically increased invasion[71, 72].

JNK-related implication for involvement of the SAPK pathway in pancreatic cancer

Increased phospho-JNK staining was observed in pancreatic cancer tissues compared to normal controls[73] and increased phospho-JNK1 staining was determined as an independent predictor of peritoneal spread[74]. Furthermore, serum auto-antibodies

against JNK2 were identified as potential biomarker in pancreatic cancer patients[75]. By isoform-specific knockdown of JNK1 and 2 in MiaPaCa-2 and Panc-1 cells, a tumor promoting role could be attributed to JNK1, while JNK2 seems to exert suppressive functions in pancreatic cancer[76]. Upstream kinases of JNK have also been studied in pancreatic cancer, but their distinct role in tumor formation and progression remains elusive. MEKK1, as a representative of JNK-activating MKKKs, was shown to contribute to pancreatic cancer cell survival. However, unlike in other cancer cell lines, JNK signaling was not affected by knockout of MEKK1 in the PDAC cell line Panc-1 [77]. MKK4, a direct upstream kinase of JNK, was expressed in the majority of resected specimens, while expression levels were reduced in matched metastatic samples. As further hints for a potential tumor suppressive role, patients with loss of MKK4 were associated with shorter survival, and pancreatic cancer cell lines frequently showed loss-of-heterozygosity for MKK4[78,79]. However, ectopic expression of MKK4 stimulated proliferation and migration of ASPC-1 and BxPC3 cells[80].

Both kinases also act in pancreatic cancer outside neoplastic cells. *Ptf1a*$^{Cre/+}$; *Kras*$^{G12D/+}$; *JNK1*$^{-/-}$ mice showed significantly smaller tumors than their *JNK1*$^{+/-}$ counterparts. Tumors induced by transplantation of murine PDAC cells were larger in wild-type mice than in *JNK1*$^{-/-}$ mice lacking JNK signaling in stromal and immune cells. Interestingly, mice heterozygous for JNK1 showed less infiltrating $CD8^+$ T cells, possibly due to JNK-mediated downregulation of chemokine secretion of tumor-associated fibroblasts[81]. On the other hand, alternative activation of p38 through the T cell receptor in $CD4^+$ T cells resulted in more aggressive disease through secretion of pro-inflammatory cytokines like interleukin-17 and TNF-α[82].

SAPK inhibitors and their role for the treatment of pancreatic cancer

Due to the involvement of SAPKs within a variety of cellular processes and diseases, multiple researchers and the pharmaceutical industry have focused on identifying pharmacological inhibitors.

Generally, JNK small molecule inhibitors can be grouped into adenosine triphosphate (ATP)-competitive and non-ATP-competitive inhibitors. ATP-competitive inhibitors represent the majority of compounds, and most of them act as pan-JNK inhibitors, as the ATP-binding pocket is highly conserved among all three isoforms [83]. Non-ATP-competitive inhibitors target the interaction of JNK with upstream and downstream targets as well as scaffolding proteins. p38 inhibitors can also be grouped by their way of action. Similar to JNK inhibitors, many p38 inhibitors act as ATP-competitive inhibitors, either binding to the active (type 1 inhibitors) or inactive conformation of p38 (type 2 inhibitors)[84]. So far, p38 as well as JNK have been targeted in therapeutic intention for multiple pathologic conditions, like neurodegenerative diseases (*e.g.*, Alzheimer's and Parkinson's disease) and inflammatory diseases (*e.g.*, rheumatoid arthritis and inflammatory bowel disease)[85-87]. Consequently, pharmacological inhibition of both kinases has also been tested in order to explore new therapeutic strategies for the treatment of neoplastic diseases. Table 1 summarizes selective SAPK inhibitors studied in the context of cancer.

Strikingly, most studies report an antitumor effect of pharmacological SAPK inhibition. However, it needs to be noted that all inhibitors were pan-JNK inhibitors and p38α or pan-p38 inhibitors, respectively. As mentioned above, we previously reported on marked isoform-specific differences. By using genetic pathway disruption, isoform-specific tumor suppressive functions of JNK2 and p38β were detected and consequently, targeting of these isoforms might increase the risk of failure in clinical studies[76]. This effect could not be observed in our study, as pharmacological inhibition of JNK also reduced cell growth in various cell lines. Up to now, clinical studies using p38 inhibitors failed or only showed moderate success. Only one JNK inhibitor (CC-401) has been clinically evaluated for the treatment of cancer (NCT00126893), but this study has been discontinued[88,89].

In an early study, Ding *et al*[90] showed that the p38 inhibitor SB203580 [half maximal inhibitory concentration (IC_{50}) = 34 nmol/L] increases the number of Panc-1 cells in S phase as well as their proliferation. Decreased p38 activity was confirmed by detection of reduced levels of phospho-activating transcription factor. However, the same study also revealed increased phosphorylation levels of ERK1/2 and JNK[90]. Increased activation of ERK1/2, possibly as a compensation mechanism or off-target effect under SB203580 treatment, has also been shown in other reports[91,92]. Therefore, it remains unclear if the observed increased proliferation is a consequence of the loss of p38-dependent tumor suppressive actions or rather of increased ERK1/2 signaling. More recently, off-target effects of SB203580 and the closely related compound SB202190 were also described by Shanware and colleagues[93], reporting on cellular effects interestingly caused by off-target inhibition of CK1. Similar to the

Table 1 Small molecule inhibitors of stress-activated protein kinases tested for treatment effects on pancreatic carcinoma cells *in vitro* and *in vivo*

Inhibitor	IC_{50} (μmol/L)	Observed effects in cell culture and *in vivo* data	Ref.
JNK inhibitor II(SP600125)	0.040 (JNK1); 0.040 (JNK2); 0.090 (JNK3)	Antitumor effects in cancer cell lines of thyroid, stomach, lung, colon, pancreas, and brain	[104, 185-189]
JNK inhibitor XVI(JNK-IN-8)	0.005 (JNK1); 0.019 (JNK2); 0.980 (JNK3)	Covalent binding to JNK inactivates kinase function; Sensitizes pancreatic cancer cells and triple negative breast cancer cells to 5-FU/FOLFOX and triple negative breast cancer cells to lapatinib treatment	[190-192]
Bentamapimod(AS602801)	0.080 (JNK1); 0.090 (JNK2); 0.230 (JNK3)	Cytotoxic effects observed on cancer stem cells derived from pancreatic cancer, non-small cell lung cancer, ovarian cancer, and glioblastoma	[103, 193]
SB203580	0.034 (p38)	Synergistic effects observed in combination with cisplatin *in vitro* and *in vivo*; Inhibition of gemcitabine-induced apoptosis in combination therapy (tested on PK-1 and PCI-43 PDAC cell lines); $IC_{50(p38)}$ = 0.08-0.20 μmol/L *in vivo*)	[194-198]
SB202190	0.050 (p38α); 0.100 (p38β2); 0.600 (CK1)	Inhibition of gemcitabine-induced apoptosis in combination therapy (tested on PK-1 and PCI-43 PDAC cell lines); Inhibits resistance of colon cancer cell lines towards irinotecan	[93,197, 199,200]
SB239063	0.044 (p38α and β)	Dose-dependent growth inhibition observed in three pancreatic cancer cell lines	[68,201]

CK1: Casein kinase 1; EC_{50}: Half maximal effective concentration; 5-FU: 5-fluorouracil; IC_{50}: Half maximal inhibitory concentration; JNK1/2/3: c-Jun N-terminal kinases 1, 2, and 3.

above mentioned studies, Zhong *et al*[68] reported on the growth enhancing effects not only for SB203580 but also for SB202190 and SB239063 on three different pancreatic cancer cell lines. Interestingly, while environmental stressors (hypoxia and reduced serum levels) led to reduced proliferation in PDAC cell lines, p38 inhibition abolished these effects. Again, increased phospho-JNK levels after p38 inhibition were reported, and JNK inhibition through SP600125 abolished the effects of p38 inhibition *in vitro*. The pan-JNK inhibitor SP600125 ($IC_{50(JNK1)}$ = 40 nmol/L; $IC_{50(JNK2)}$ = 40 nmol/L; $IC_{50(JNK3)}$ = 90 nmol/L) also reduced the *in vivo* growth of cell lines with high phospho-p38 Levels [68]. The crosstalk of p38 and JNK has been described in various contexts previously. Although there is evidence for a synergistic role of both SAPKs in activation of downstream targets[94], an opposing function of both has also been well documented [95-97]. Possible regulation mechanisms of JNK through p38 include upstream MKKKs (MLK3, TAK1) as well as nuclear factor-κB[44]. Consequently, targeting single MAPKs is highly challenging. An alternative approach to using inhibitors could be selectively activating pathways of interest. The small molecule triptonide was shown to selectively activate the MEKK4-MKK4-p38 pathway without significantly altering phosphorylation levels of JNK and ERK1/2. This resulted in dose-dependent growth reduction of six pancreatic cancer cell lines as well as *in vivo* xenografts by inducing G2/M arrest and reduced expression of cyclin-dependent kinase 3[98].

In contrast to those studies showing an overall growth restraining effect of p38 in pancreatic cancer, Yang *et al*[99] performed a screen of p38α expression in various cancer samples of The Cancer Genome Atlas database and identified an overexpression of p38α in PDAC samples. The same study also reported enhanced phospho-p38 labeling in PDAC tissues compared to adjacent normal tissue and mostly attributed phospho-p38 labeling to cancer cells. When treating Pan02 cells with SB203580 or the p38α- and β-specific inhibitor LY2228820, Yang *et al*[99] reported growth-restricting effects. However, it needs to be noted that the used inhibitor concentrations were relatively high and potential off-target effects cannot be excluded. Finally, in order to address the issue of lacking sensitivity, possible binding pockets in p38, enabling the design of more selective inhibitor compounds in future, were identified by *in silico* modeling[99].

Previous studies indirectly suggested a growth-promoting effect of JNKs on pancreatic cancer. Takahashi *et al*[73] observed growth inhibition *in vitro* and *in vivo* after treatment with SP600125. This was associated with G1 arrest and downregulation of cyclin D1 *in vitro*. In genetically-engineered mouse models (*Ptf1a*$^{cre/+}$, *LSL-Kras*$^{G12D/+}$, and *Tgfbr2*$^{flox/flox}$) SP600125 reduced neoangiogenesis and expression levels of CD44 in PDAC cells[73]. Together with CD133, CD44 is considered as a potential marker for cancer stem cells (CSCs) or CSC-like cells (CSCLCs)[100,101]. Increased levels of phospho-JNK was also shown in CSCLCs of pancreatic cancer and other human malignancies[102]. Inhibition of JNK by the pan-JNK inhibitors SP600125 or AS602801 as well as genetic targeting of JNK1 and 2 *via* small interfering RNA-mediated knockdown reduced levels of $CD133^+$ cells in an isolated subpopulation of pancreatic

cancer-derived CSCLCs and abolished their self-renewal capacity *in vitro* and *in vivo* [102,103]. Besides growth suppression and interference with CSCs, induction of cellular differentiation can be another mechanism of JNK inhibition-mediated tumor suppression[104].

In clinical practice, SAPK inhibition will rather be used for combination therapy approaches instead of single-agent therapy. Therefore, the interference of SAPK inhibitors with standard of care chemotherapeutics is highly relevant. In addition to single treatment of CSCLCs, the group of Suzuki *et al*[105] also investigated the effects of JNK inhibition in combination with gemcitabine and 5-fluorouracil (5-FU). While CSCLCs expectedly were more resistant to these agents, pretreatment with SP600125 had a synergistic effect in combination with gemcitabine and 5-FU in a reactive oxygen species-based way of action[105]. The observed synergistic effect can furthermore be explained by JNK-mediated effects on multidrug resistance. Multidrug resistance is not only a hallmark of CSCs but of cancer cells in general, and multidrug transporters like P-glycoprotein reduce intracellular drug levels[106]. In this context, high JNK levels were shown to decrease P-glycoprotein levels in pancreatic and gastric cancer, thereby increasing intracellular drug concentrations as well as drug sensitivity[107].

The interplay of the p38 pathway and gemcitabine treatment has been well studied in pancreatic cancer cells. Apoptosis mediated through gemcitabine was consistently associated with p38 activation as well as caspase-dependent cleavage of poly (ADP-ribose) polymerase and heat shock protein 27 phosphorylation. Inhibition of p38 by SB203580 reversed these effects. Similarly, inhibition of MAPK-activated protein kinase 2, a downstream target of p38, abolished gemcitabine-mediated apoptosis in pancreatic cancer. However, combination of p38 inhibitors with mitomycin C showed synergistic effects[108].

PROTEIN KINASE CK1 FAMILY

In 1954 for the first time, an enzyme was isolated from liver tissue, which was able to phosphorylate the milk protein casein[109]. Fifteen years later, two distinct protein kinases with the ability to phosphorylate casein (at least *in vitro*) were described and termed casein kinase 1 (CK1) and casein kinase 2 (CK2), meanwhile renamed protein kinases CK1 and CK2[110]. Despite their common nomenclature and the ability to phosphorylate casein, protein kinases CK1 and CK2 are highly different with respect to their classification and cellular functions. While CK2 belongs to the CMGC [containing cyclin-dependent kinase, MAPK, glycogen synthase kinase 3 (GSK3), and cdc2-like kinase families] group of the human kinome, CK1 forms an independent family of protein kinases[111].

In the human genome, six CK1 isoforms are encoded (α, γ1, γ2, γ3, δ, and ε), and several splice variants can originate from post-transcriptional processing. While all CK1 isoforms are highly conserved within the kinase domain, the sequences of the N- and C-terminal noncatalytic domains can be quite variable[20,112]. Although the protein kinases of the CK1 family are generally considered to be constitutively active, several regulatory mechanisms have been described. Expression and/or activity levels of CK1 isoforms can be enhanced by insulin or cellular stress executed by viral transformation, topoisomerase inhibitor treatment, or γ-irradiation. Regulation of enzymatic activity is also possible on the protein level, *e.g.*, by modulation of subcellular localization, interaction with other proteins, or (auto-)phosphorylation in particular targeting the C-terminal regulatory domain but also the kinase domain[20,112].

Most CK1 isoforms are localized in the cytosol. Only the CK1αL variant possessing a second nuclear localization signal in the L-exon can be localized to the nucleus[113]. Due to C-terminal palmitoylation, CK1γ can be associated with the plasma membrane [114-116]. By modulation of subcellular localization, CK1 isoforms can be brought in proximity with different substrate pools. Substrate recognition motifs for CK1 can generally be found on most cellular proteins and, to date, more than 150 substrates being phosphorylated by CK1 isoforms at least *in vitro* have been reported[20]. Thereby, CK1 shows strong preferences for acidic or phospho-primed substrates presenting the canonical consensus sequence (phospho-Ser/phospho-Thr-X-X-(X)-Ser/Thr). In addition, several alternative noncanonical motifs targeted by CK1 have been described[117,118].

The broad range of substrates phosphorylated by CK1 gives a hint of the numerous cellular processes potentially regulated by CK1 family members. These processes involve cell proliferation and differentiation, DNA processing and repair, as well as

cytoskeleton maintenance just to name few of them. In particular, essential signal transduction pathways involving CK1-mediated regulation include Wnt and Hedgehog (Hh) signaling as well as regulation of circadian rhythm[20]. Consequently, deregulation or dysfunction of CK1 isoforms involved in regulation of these signaling pathways can result in deregulated signal transduction and subsequent development of pathological states.

Relevant CK1-related pathways in cancer

CK1 isoforms have been implicated in several signaling pathways such as the canonical and noncanonical Wnt as well as Hh and Hippo signaling pathways, which play an important role in tissue development, growth, and homeostasis[119-122]. Aberrant signaling as well as mutations of key regulator proteins of these pathways can lead to various cancer entities[123-127]. The connection between CK1 and cancer has been strengthened through the discovery of their targets such as β-catenin, p53, and mouse double minute homologue 2 and 4, which hold important roles as key regulators in signaling pathways and are generally thought to be involved in cancer development (Figure 1)[128,129]. Considering the reported CK1-mediated phosphorylation of numerous substrates essential for signal transduction, it is not surprising that CK1-specific mutations resulting in altered expression levels or enzyme activity are likely to be accompanied by dramatic changes affecting CK1-regulated signal transduction pathways.

As one of the best characterized CK1-regulated processes, the Wnt signaling pathway has an important regulatory role in cell proliferation, differentiation, and cell polarity[120,130-133]. Altered expression levels of key regulators within the pathway are associated to oncogenesis, both through increased expression of positive regulators and decreased expression of negative regulators[134-137]. Several studies showed that all CK1 isoforms are implicated in the Wnt signaling pathway and either exert positive or negative regulatory functions, respectively[14]. Acting as positive regulators of the canonical Wnt signaling pathway, CK1γ, δ, and ε were found to initiate the transcription of proto-oncogenes like cyclin D1 and c-myc resulting in increased cell proliferation and cell survival[13,138,139]. For instance, mutations within the C-terminal region of CK1δ were shown to alter its physiological role, increase the oncogenic potential, and promote colonic adenoma development[140]. Additionally, CK1 isoforms exhibit oncogenic characteristics associated to the inhibition of apoptotic processes. This assumption is supported by the findings that CK1δ and CK1ε contribute to the switching mechanism between the canonical and the non-canonical Wnt/Rac1/JNK pathway, where they may favor the canonical Wnt pathway to the detriment of JNK-mediated apoptosis[17,141]. In many Wnt-driven cancers, CK1α protein expression is suppressed, leading to an activation of proliferative processes *via* the Wnt pathway. In addition, the absence of CK1α leads to a critical involvement of p53 in controlling invasiveness, which was shown in a model for colon cancer[15].

The importance of CK1 isoforms within various signaling pathways is strengthened by reports linking CK1 to phosphorylation of components in Hh signaling pathway. Although the activity of the Hh signaling pathway is reduced in adulthood, it is critical for embryonic development, organogenesis, and maintenance of healthy adult cells[119]. In the adult organism, Hh signaling contributes to the regulation of epithelial maintenance and tissue regeneration; consequently mutations and dysregulation of components of this signaling pathway promote tumorigenesis and cancer development[142-145]. As seen in Wnt signaling, CK1 isoforms appear to have contrasting effects on Hh signaling. Acting as a negative regulator, CK1 promotes proteolysis of GLI TF and prevents target gene transcription[146-148]. In order to fulfill its positive function, CK1α and G-protein coupled receptor kinase 2 phosphorylate the positive Hh regulator Smoothened homologue precursor, thereby inducing its active conformation[149].

The major functions of the Hippo pathway have been defined to correct organ maturation through restriction of organ size by regulating cell proliferation and apoptosis[150]. As such, dysregulated Hippo signaling can trigger tumorigenesis and cancer. CK1 isoforms have been proposed to regulate Hippo signaling through phosphorylation of a phosphodegron signal in Yes-associated protein after receiving priming phosphorylation by large tumor suppressors 1 and 2. As a result, the phospho-degron signal mediates recruitment of βTrCP ubiquitin ligase causing Yes-associated protein degradation and inhibition of cell growth and differentiation[150]. Additionally, a more recent publication proposed an interaction between the Wnt and the Hippo pathways mediated through CK1ε. In this context, the Hippo upstream kinase MST1 inhibits the Wnt signaling pathway by directly binding CK1ε and thereby suppressing phosphorylation of Disheveled[151].

Implication for CK1 involvement in pancreatic cancer

In a recent study analyzing messenger RNA-based gene expression data of the International Cancer Genome Consortium Pancreatic Cancer Australia cohort, high expression levels of CK1δ detected in patients with pancreatic cancer were correlated with poor survival. Increased expression of CK1δ could be found in patients with metastatic pancreatic carcinoma, and CK1δ expression was furthermore strongly correlated with the tumor grade[152]. This observation is in line with previous studies reporting upregulation of CK1 isoforms in PDAC in general[153,154] and describing increased expression of CK1δ and CK1ε in a patient cohort with higher-graded PDAC [155]. Cell line-specific elevated expression levels of CK1δ and/or CK1ε were also detected in various tumor cell lines[152,155]. Independent of the detected protein levels, CK1δ- and CK1ε-specific kinase activities in extracts obtained from various pancreatic cancer cell lines (MiaPaCa-2, BxPC3, PancTu-1, and Colo357) significantly differed from each other by up to six orders of magnitude[156].

In general, due to the involvement of CK1 isoforms in various pathways related to tumorigenesis, altered expression and/or activity levels of CK1 isoforms can be associated with increased oncogenic potential. Using the breast cancer cell line MCF7, a regulatory function of CK1ε has been identified in the Akt pathway[157]. This is of particular interest because Akt is frequently upregulated in PDAC[158,159]. In detail, CK1ε is able to inhibit protein phosphatase 2B, consequently resulting in increased Akt phosphorylation levels and enhanced Akt kinase activity. Inhibition of CK1ε in MCF7 cells by the small molecule inhibitor IC261 has been demonstrated to reduce Akt phosphorylation as well as Akt-mediated phosphorylation of GSK3β[157]. Quite similar findings could be made using PDAC cells. Also in this case, phosphorylation of Akt was reduced in response to treatment with IC261[160]. However, these results were only based on observations made in preliminary experiments, and effects were obtained by using extremely high concentrations of the rather unspecific early-stage inhibitor IC261.

Apart from altered expression and/or activity levels, mutations in the coding sequence for CK1 isoforms can also be associated with increased oncogenic functions of the resulting CK1 mutant proteins. Several mutations in *CSNK1D*, the gene coding for human protein kinase CK1δ, identified in different types of cancer (*e.g.*, colorectal carcinoma, lung squamous cell carcinoma, bladder urothelial carcinoma, and pancreatic carcinoma) were analyzed for their enzyme kinetic parameters and their sensitivity towards the treatment with several CK1-specific small molecule inhibitors. Among the tested mutants, hyperactive (*e.g.*, R127L and R127Q) as well as nearly inactive (*e.g.*, E247K and L252P) variants could be characterized. Especially, the hyperactive CK1δ mutant R127Q showed enhanced sensitivity towards the treatment with various CK1-specific inhibitors. The two tested CK1δ mutants exclusively detected in PDAC (Q399* and H414Y) only showed slightly reduced kinase activity when compared to wild-type CK1δ[161]. The online analysis tool cBioPortal for Cancer Genomics lists even more mutations detected in PDAC and affecting CK1 isoforms, among them frameshift deletions as well as nonsense and missense mutations[162-165]. However, these mutations have so far not been investigated for their oncogenic potential.

Unfortunately, no detailed information on the role of CK1 isoforms in formation of metastasis from primary tumors located in the pancreas has been available so far. In general, the zinc-finger TF Snail is phosphorylated by CK1ε and GSK3β in a hierarchical manner. Snail can promote epithelial-mesenchymal transition by repressing expression of E-cadherin but is degraded by the proteasome upon phosphorylation by CK1ε and GSK3β. Pharmacological inhibition (using the inhibitor IC261) or RNA interference-mediated downregulation of CK1δ inhibits phosphorylation of Snail and promotes cell migration[166]. In addition, reduced proliferation and invasion could be linked with CK1ε-mediated inhibition of Wnt/β-catenin signaling and downregulation of Wnt3a, β-catenin, proliferating cell nuclear antigen, and matrix metalloproteinase 9 in colorectal cancer cells[167].

Inhibitors of CK1 and their role for the treatment of pancreatic cancer

Results obtained from numerous studies conducted within the last 10-15 years characterized the protein kinases of the CK1 family as well-established drug targets. While early-stage small molecule inhibitors (*e.g.*, IC261[168]) only demonstrated low target selectivity, several recent-stage CK1-specific inhibitors with enhanced selectivity and improved potency in the nanomolar range are available to date (Table 2)[20,112,169]. So far, none of these inhibitors advanced to the stage of clinical trials, and the use of these compounds was limited to biochemical and cell culture-based testing or animal

Table 2 Casein kinase 1-specific small molecule inhibitors tested for treatment effects on pancreatic carcinoma cells *in vitro* and *in vivo*

Inhibitor	IC_{50} CK1 (μmol/L)	Observed effects in cell culture and *in vivo* data	Ref.
IC261	1.000 ± 0.30 (CK1δ)	Reduced growth of ASPC-1, BxPC3, Capan-1, Colo357, MiaPaCa-2, Panc-1, Panc89, and PancTu-1 at 1.25 μmol/L concentration of IC261; Subcutaneous xenograft model using PancTu-2: reduced tumor size with IC261 or gemcitabine (no synergism with gemcitabine), downregulation of anti-apoptotic genes/upregulation of cell cycle- and cell death-associated regulators; Notable off target effects (affecting the cytoskeleton and ion channels)	[155, 168, 182-184, 202]
compound 11b	0.004 ± 0.001 (CK1δ); 0.025 ± 0.004 (CK1ε); 0.010 (p38α)	Cytotoxic effects observed on Colo357 (EC_{50} = 3.5 ± 0.3 μmol/L) and Panc89 (1.5 ± 0.4 μmol/L)	[174]
compound 3c	1.600 (CK1δ/ε)	In a panel of cell lines only effective against Panc-1 (EC_{50} = 9.3 ± 0.0 μmol/L); Cytotoxic effects observed on A549 (lung carcinoma) and Hek293 (normal cells) significantly higher EC_{50} values	[175]
compound 2	0.070 ± 0.01 (CK1δkd); 0.520 ± 0.05 (CK1ε)	Cytotoxic effects observed on BxPC3 (EC_{50} = 0.11 ± 0.01 μmol/L), Colo357 (0.13 ± 0.02 μmol/L), MiaPaCa (0.26 ± 0.02 μmol/L), PancTu-1 (0.70 ± 0.02 μmol/L), and Panc-1 (0.35 ± 0.08 μmol/L); Cell line-specific effects observed in screening against a panel of 82 tumor cell lines	[178]
IWP-4	1.020 ± 0.13 (CK1δ); 7.070 ± 2.01 (CK1ε)	Cytotoxic effects observed on A818-6 (EC_{50} = 0.93 ± 0.07 μmol/L), MiaPaCa-2 (0.23 ± 0.01 μmol/L), Panc-1 (0.23 ± 0.02 μmol/L), Panc89 (0.58 ± 0.12 μmol/L), and Capan (0.23 ± 0.01 μmol/L); Inhibition of Wnt signaling (Wnt3A overexpression, autocrine/paracrine) with IC_{50} = 0.71 ± 0.38 μmol/L; Inhibition of Wnt signaling (Wnt3A-conditioned medium, autocrine/paracrine) with EC_{50} = 1.47 ± 0.55 μmol/L	
SR-3029	0.044 (CK1δ); 0.260 (CK1ε)	Cytotoxic effects observed on Panc-1 (EC_{50} = 0.023 μmol/L), MiaPaCa2 (0.370 μmol/L), and BxPC3 (0.131 μmol/L); Mouse pharmacokinetic studies with promising results for animal model use of SR-3029; Orthotopic xenograft model using Panc-1, reduced tumor size using SR-3029 and/or gemcitabine (synergism with gemcitabine due to upregulation of dCK)	[152, 180]

CK1: Casein kinase 1; EC_{50}: Half maximal effective concentration; IC_{50}: Half maximal inhibitory concentration.

models.

Quite recently, we characterized optimized 4,5-diarylimidazoles as highly effective ATP-competitive inhibitors of CK1δ. Substituted isoxazoles were originally designed as inhibitors of p38α MAPK, but they share the same pharmacophore moiety that is necessary to inhibit CK1δ[93,170-172]. Substituting the isoxazole scaffold with an imidazole scaffold resulted in the generation of highly potent dual-specific inhibitors of p38α MAPK and CK1δ[173]. By further optimizing these imidazole-based compounds, CK1 isoform-specific inhibitors with IC_{50} values in the low nanomolar range like compounds 11b [$IC_{50(CK1\delta)}$ = 4 nmol/L], 12a (19 nmol/L), and 16b (8 nmol/L) could be developed, which represent the most potent CK1δ-specific inhibitors described so far. Because IC_{50} values determined for the highly related isoform CK1ε are increased by six to 12 orders of magnitude (with 25, 227, and 81 nmol/L for 11b, 12a, and 16b, respectively), these compounds can also be considered to be selective for CK1δ. Compound 11b even demonstrated superior selectivity towards CK1δ among a panel of more than 321 protein kinases. However, full selectivity with respect to side-effects on p38α MAPK could still not be achieved for this set of compounds, but IC_{50} values determined for p38α are three-fold higher compared to CK1δ. Finally, 11b demonstrated significant effects on pancreatic cancer cell lines, with half maximal effective concentration (EC_{50}) values in the low micromolar range [$EC_{50(Colo357)}$ = 3.5 μmol/L, $EC_{50(Panc89)}$ = 1.5 μmol/L)[174].

Apart from isoxazole- and imidazole-derived molecules, the quinazoline-based inhibitors (N-(1H-pyrazol-3-yl)quinazolin-4-amines) 3c and 3d have been shown to inhibit CK1δ and ε ($IC_{50(CK1\delta/\varepsilon)}$ = 1.6 and 1.4 μmol/L, respectively). In a panel of human cancer cell lines, compound 3c even demonstrated selective cytotoxicity against the PDAC cell line Panc-1, with an EC_{50} value of 9.3 μmol/L [for all others no EC_{50} value could be determined (> 100 μmol/L), except for A549 with 29.7 and HEK293 with 71.1 μmol/L]. Compound 3d also demonstrated effects on Panc-1 cells but only with an extremely high EC_{50} value of 69.4 μmol/L. However, the mechanism of selectivity of the tested quinazoline-based inhibitor remains to be determined[175].

Within the last decade, several benzimidazole-based inhibitors have demonstrated significant inhibition of CK1δ variants and superior isoform selectivity over CK1δ. The series of compounds described by Leban *et al*[176] originates from piperidinyl-thiazoles originally designed to inhibit nuclear factor-κB[176]. Following modification, these compounds also demonstrated significant inhibition of CK1 family members. Most significant inhibition of CK1δ kinase domain (CK1δkd) with superior isoform selectivity over CK1ε could be determined for compound 5 ($IC_{50(CK1\delta kd)}$ = 29 nmol/L, $IC_{50(CK1\varepsilon)}$ = 199 nmol/L). Compound 5 also induced apoptosis in various tumor cell lines

with cell line-specific effects and only moderate levels of apoptosis in Colo357 pancreatic cancer cells (tested at 4 μmol/L concentration)[177]. As reported by Richter and colleagues[178], the highly related but structurally slightly different compound 1 showed three-fold stronger inhibition of CK1δkd (IC_{50} = 10 nmol/L). By further improving the physicochemical properties of this difluoro-dioxolo-benzoimidazole derivative, inhibitor potency *in vitro* could be maintained for modified compound 2 ($IC_{50(CK1\delta kd)}$ = 0.07 μmol/L, $IC_{50(CK1\varepsilon)}$ = 0.52 μmol/L) while significantly increasing the effects observed on a panel of cancer cell lines. In comparison to compound 1, the effects on cell viability were significantly increased for cell lines treated with compound 2, among them the pancreatic cancer cell lines BxPC3, Colo357, MiaPaCa, PancTu-1, and Panc-1 (see Table 2 for EC_{50} data)[178].

Being structurally related to benzimidazole-based inhibitors, compounds derived from inhibitors of Wnt production (IWP) have recently been described as CK1-specific inhibitors. IWP-2 and IWP-4 as well as the further optimized compound 19 displayed rather potent inhibition of CK1δkd *in vitro* ($IC_{50(CK1\delta)}$ = 0.32, 1.02, and 0.09 μmol/L for IWP-2, IWP-4, and compound 19) and also demonstrated significant effects on the proliferation of pancreatic cancer cell lines as determined for IWP-4-treated A818-6 (EC_{50} = 0.93 μmol/L), MiaPaCa (0.23 μmol/L), Panc-1 (0.23 μmol/L), and Panc89 (0.58 μmol/L) cells[179].

As a benzimidazole-based inhibitor containing a purine scaffold compound SR-3029 has been described as highly potent and selective inhibitor of CK1δ ($IC_{50(CK1\delta)}$ = 44 nmol/L, $IC_{50(CK1\varepsilon)}$ = 260 nmol/L)[180]. SR-3029 shows improved cellular activity on the human melanoma cell line A375 (EC_{50} = 86 nmol/L) and the triple-negative breast cancer cell line MDA-MB-231[181]. These results suggested favorable cell penetration for SR-3029, and mouse pharmacokinetic properties indicated that SR-3029 actually was sufficient for use in xenograft studies[180].

Recently, SR-3029 has been tested for its effects on the proliferation of PDAC cell lines Panc-1, MiaPaCa-2, and BxPC3, thereby obtaining EC_{50} values in the submicromolar range (23, 370, and 131 nmol/L, respectively). Furthermore, synergistic effects have been detected for the treatment of MiaPaCa-2 and Panc-1 cells with a combination of SR-3029 and gemcitabine, the standard of care used in treatment of locally advanced and metastatic PDAC. Same effects could be observed after silencing of CK1δ by small interfering RNA. The mechanism of synergy could be explained by upregulation of deoxycytidine kinase subsequent to inhibition of CK1δ by SR-3029, resulting in enhanced metabolism and anti-proliferative effects of gemcitabine. Anti-proliferative effects of SR-3029 and synergy with gemcitabine could also be observed *in vivo* by using an orthotopic xenotransplantation mouse model. Tumors obtained from injection of Panc-1 cells into the pancreas were significantly smaller after treatment with SR-3029 or gemcitabine, and tumor size was even more reduced after combination therapy[152].

In a previous xenotransplantation study, the early-stage CK1-specific inhibitor IC261 had already demonstrated therapeutic potential. Tumor cell growth of a panel of established pancreatic cancer cell lines (ASPC-1, BxPC3, Capan-1, Colo357, MiaPaCa-2, Panc-1, Panc89, and PancTu-1) was significantly reduced by treatment with 1.25 μmol/L IC261 *in vitro*, and the size of tumors obtained after subcutaneous injection of PancTu-2 cells was significantly smaller after treatment with IC261. In the tumor tissue, downregulation of several anti-apoptotic genes (*e.g.*, Bcl-2 family members) and upregulation of cell cycle- and cell death-associated regulators (*e.g.*, p21, ataxia-telangiectasia mutated kinase, checkpoint kinase 1) could be observed following treatment with IC261 or gemcitabine[155]. However, and in contrast to the above mentioned recent study by Vena and colleagues[152], IC261 failed to sensitize gemcitabine-resistant PancTu-1 cells to treatment with gemcitabine, and no synergistic or additive action in combination with gemcitabine could be demonstrated for IC261. This failure can be due to the unspecific effects meanwhile described for IC261. Apart from its specific action on CK1 family members, IC261 is able to bind tubulin with an affinity similar to the spindle poison colchicine. IC261 can therefore be considered as a microtubule polymerization inhibitor by directly exerting its effects on microtubules independent of CK1 blockage[182,183]. Moreover, within the concentration range necessary to block CK1 kinase activity, IC261 is also able to block voltage-gated sodium channels, and consequently, well-characterized recent-stage CK1-specific inhibitor compounds like SR-3029 should be used for targeting CK1 isoforms instead of using the unspecific early-stage inhibitor IC261[184].

CONCLUSION

In recent years, there has been a lot of evidence for the involvement of stress-activated kinases like JNK and p38 but also CK1 in the pathogenesis of pancreatic cancer. Furthermore, remarkable progress has been made in designing specific small molecule inhibitors to effectively target these kinases *in vitro* and *in vivo* and to reduce off-target effects. Interestingly, due to similarities in protein structure, some inhibitor compounds even demonstrate dual inhibition of p38 and CK1 isoforms. However, further mechanisms and benefits from dual kinase inhibition have not been studied in detail. Furthermore, conclusive results from using specific inhibitors in clinical trials remain to be obtained, and knowledge on the interplay of these inhibitors with standard of care chemotherapeutics needs to be acquired in future studies.

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REVIEW

Malnutrition and liver disease in a developing country

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Abstract

Malnutrition is a highly prevalent and under recognized condition in developing countries of South Asia. The presence of malnutrition causes a severe impact on patients with liver cirrhosis. The etiology of cirrhosis differs in the South Asian region compared to the West, with hepatitis B and C still being the leading causes and the prevalence of nonalcoholic fatty liver disease increasing over time. Comorbid malnutrition worsens outcomes for cirrhosis patients. Urgent attention to address malnutrition is needed to improve patient outcomes. The etiology and pathophysiology of malnutrition in liver diseases is multifactorial, as reduction in liver function affects both macronutrients and micronutrients. A need for nutritional status assessment for liver disease patients exists in all parts of the world. There are many widely studied tools in use to perform a thorough nutritional assessment, of which some tools are low cost and do not require extensive training. These tools can be studied and evaluated for use in the resource limited setting of a country like Pakistan. Treatment guidelines for proper nutrition maintenance in chronic liver disease exist for all parts of the world, but the knowledge and practice of nutritional counseling in Pakistan is poor, both amongst patients and physicians. Emphasis on assessment for nutritional status at the initial visit with recording of vital signs is needed. Simultaneously, treating physicians need to be made aware of the misconceptions surrounding nutritional restrictions in cirrhosis so that patient education is done correctly based on proper scientific evidence.

Key Words: Liver cirrhosis; Malnutrition; Nutrition assessment; Liver diseases; Nutritional and metabolic diseases; Micronutrients

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Core Tip: Malnutrition in liver cirrhosis is a serious problem in South Asia where the etiology differs from the Western population. As malnutrition is generally highly prevalent in the region, it causes an impact on patients with liver cirrhosis. Urgent attention to address malnutrition is needed to improve patient outcomes. Emphasis on assessment for nutritional status at the initial visit with recording of vital signs is also needed. Simultaneously, treating physicians need to be made aware of the misconceptions surrounding nutritional restrictions in cirrhosis so that patient education is done correctly based on proper scientific evidence.

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INTRODUCTION

Malnutrition (undernutrition) is defined as an insufficient intake or assimilation of nutrients essential for development and prevention of disease[1]. Malnutrition presents as a common complication of end-stage liver failure (cirrhosis), which is characterized by mass systemic complications such as refractory ascites, spontaneous bacterial peritonitis, hepatorenal syndrome and variceal hemorrhage[2]. Due to the deterioration of health that cirrhosis causes, approximately a million people die from cirrhosis globally, making it a significant disease burden to be addressed.

Several studies have evaluated nutritional status in patients with liver cirrhosis of different etiologies and varying degrees of liver insufficiency[3,4] leading to a consensus that malnutrition is recognizable in all forms of cirrhosis[5] with the prevalence of malnutrition in cirrhosis having been estimated to range from 65%-100%[6,7]. Thus, malnutrition becomes an important prognostic indicator of clinical outcomes, which include survival rate, the length of hospital stay, post-transplantation morbidity and quality of life in patients with cirrhosis[8], particularly in patients with severe cirrhosis and alcoholic cirrhosis where malnutrition is more likely to be present[9]. While malnutrition remains potentially modifiable, it has been observed starting from the initial stages of liver disease that results in an almost direct relationship between the liver disease severity and the extent of malnutrition, with malnutrition becoming easier to detect in severe cirrhosis[10].

The causes of malnutrition in liver disease are complex and multifactorial due to the liver forming a fundamental part of the body's metabolism. It results from impaired dietary intake due to the malfunctioning liver, chronic inflammation and subsequently the altered macronutrient and micronutrient metabolism. Low patient activity is also attributed to it[7,11]. Additionally, anorexia, gastroparesis, nausea, increased leptin levels, encephalopathy and gastritis are found to be contributors of malnutrition. Ascites, frequent paracentesis, some drugs such as diuretics and lactulose, a sodium-restricted diet and alcohol consumption (decreases appetite) can also result in metabolic malfunctioning and thus contribute to a reduction in dietary intake, which leads to malnutrition in liver disease[12,13]. The deficiency of biliary salts due to liver malfunction and decreased assimilation of nutrients, abnormal motility of the digestive tract and an increased bacterial growth may subsequently lead to an impaired uptake and metabolism of nutrients, resulting in malnutrition[14]. Portal hypertension leads to an increased permeability of the intestinal mucosa, thereby causing an increased loss of proteins that may be similarly noted in cases of bleeds caused by varices or ulcers[15]. A poor nutritional status then poses significant consequences for postoperative complications among transplant candidates as well because this is an important prognosticator for mortality and morbidity among chronic liver disease (CLD) patients[9]. Subsequently, malnutrition itself becomes an independent predictor of mortality in patients with CLD.

The resultant malnutrition comprises of both macro and micronutrient deficiencies. The macronutrients (being proteins, carbohydrates and fats) have their metabolism affected[16], while protein deficiency makes patients more susceptible to hepatocellular disease, in particular. On the micronutrient level, patients with a history of cholestatic liver disease are more liable to have an insufficient calorie intake with a

higher risk of vitamin deficiencies. Detecting micro or macronutrient deficiencies at an earlier stage is crucial so that nutritional supplementation can be initiated in a timely manner, which has been proven to decrease the risk of infection and in-hospital mortality while enhancing liver function. Therefore, it has been recommended that all patients with CLD should be screened to have an early identification of those at risk of complications so that relevant interventions can be started[17].

In this article, we will be reviewing the prevalence, underlying mechanism and factors associated with malnutrition and the impact of malnutrition in cirrhosis in the setting of a developing country. This will pave the way for increased awareness regarding malnutrition in cirrhosis and its consequences to subsequently treat the patients accordingly for better outcomes.

MALNUTRITION IN SOUTH ASIA

While about 2 billion people in the world are suffering from various forms of malnutrition[18], the prevalence of malnutrition due to cirrhosis is estimated to range from 65%-100% globally[6,7]. In South Asia particularly, malnutrition is rampant with a high prevalence of low adult body mass index (BMI) (23.4% in men and 24.0% in women in 2014)[19]. In this region, Pakistan in particular has 45.3 million people (28 percent of the population) being victims of food insecurity according to the National Nutrition Survey Report 2011[20]. The global nutrition report 2020 has studied the progress of attaining 10 global nutrition targets of 2025 in 194 countries (which include anemia, low birth weight, exclusive breastfeeding, childhood stunting, childhood wasting, childhood overweight, adult obesity and adult diabetes, salt intake and raised blood pressure). Progress is not assessed at the country level for salt intake and blood pressure. Data collected for 8 of the 10 targets shows that Pakistan is on track for only 2 targets out of 8. In Pakistan, more than half of children grow up stunted or wasted. Pakistan is recognizing this issue as important, as is evident by it being 1 of the 25 countries to have revisited their nutrition budget, which has resulted in allocating increased funds towards nutrition thrice since 2015. Of this, the highest increase is towards nutrition sensitive allocation of social protection. The report highlights the importance for subnational level of financing for nutrition in areas like Balochistan in Pakistan and Rajhasthan in India. Pakistan and India comprise two of the three countries with the highest number of stunted children with 10.7 million stunted children in Pakistan and 25.5 million in India. This data reflects the severity of malnutrition in the region and can be extrapolated to the adult population of the South Asian countries[21].

LIVER DISEASES IN SOUTH ASIA

While most of the burden of liver cirrhosis in western countries is nonalcoholic fatty liver disease, most of it for South Asian countries is related to hepatitis B and C. However, increasing prevalence of nonalcoholic fatty liver disease is being seen in the South Asian population as well[22]. A survey conducted in 2015 showed the incidence of cirrhosis in South Asia to be 21.39% overall with 6.98% being hepatitis B virus related, 4.87% hepatitis C virus related, 4.84% alcohol related and 4.69% due to other etiologies[23]. The incidence of cirrhosis is estimated to be 23.6 per 100000 in Southeast Asia in 2017[24,25]. A study comparing liver resection patients between the East and the West of the world showed that patients from the East had worse Child Pugh scores and had a more advanced stage of cancer than the West[26]. A multicenter study from India revealed that at least a third of the patients presenting with CLD present at a very advanced stage of decompensated cirrhosis[27].

ETIOLOGY AND PATHOPHYSIOLOGY

The flowcharts in Figures 1 and 2 describe the pathophysiology of malnutrition as a consequence of cirrhosis and the multiple pathways that lead to it[28].

Metabolic changes occurring in CLD

Multiple changes in nutrient metabolism are observed in cirrhosis, which subsequently contributes to malnutrition. Around 14% to 40% of patients with cirrhosis

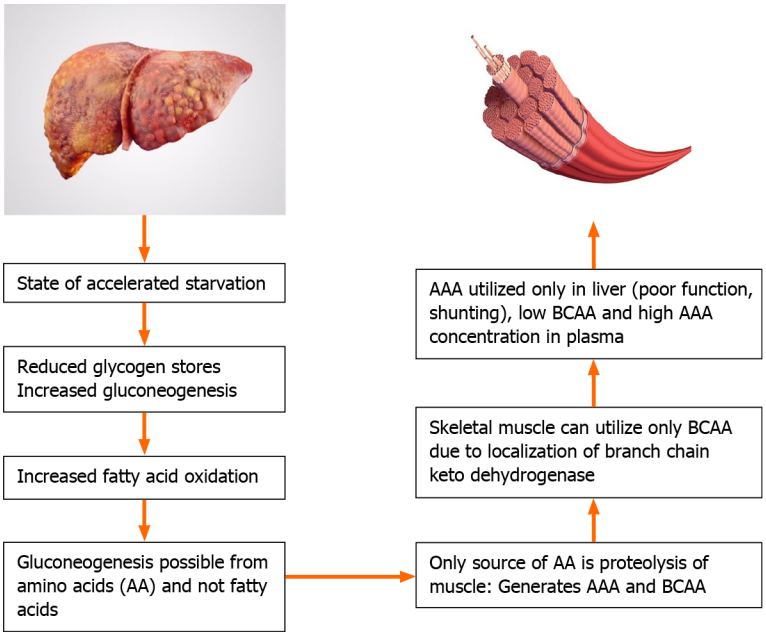


Figure 1 Pathophysiology of malnutrition as a consequence of cirrhosis and the multiple pathways that lead to it. AAA: Aromatic amino acids; BCAA: Branched chain amino acids.

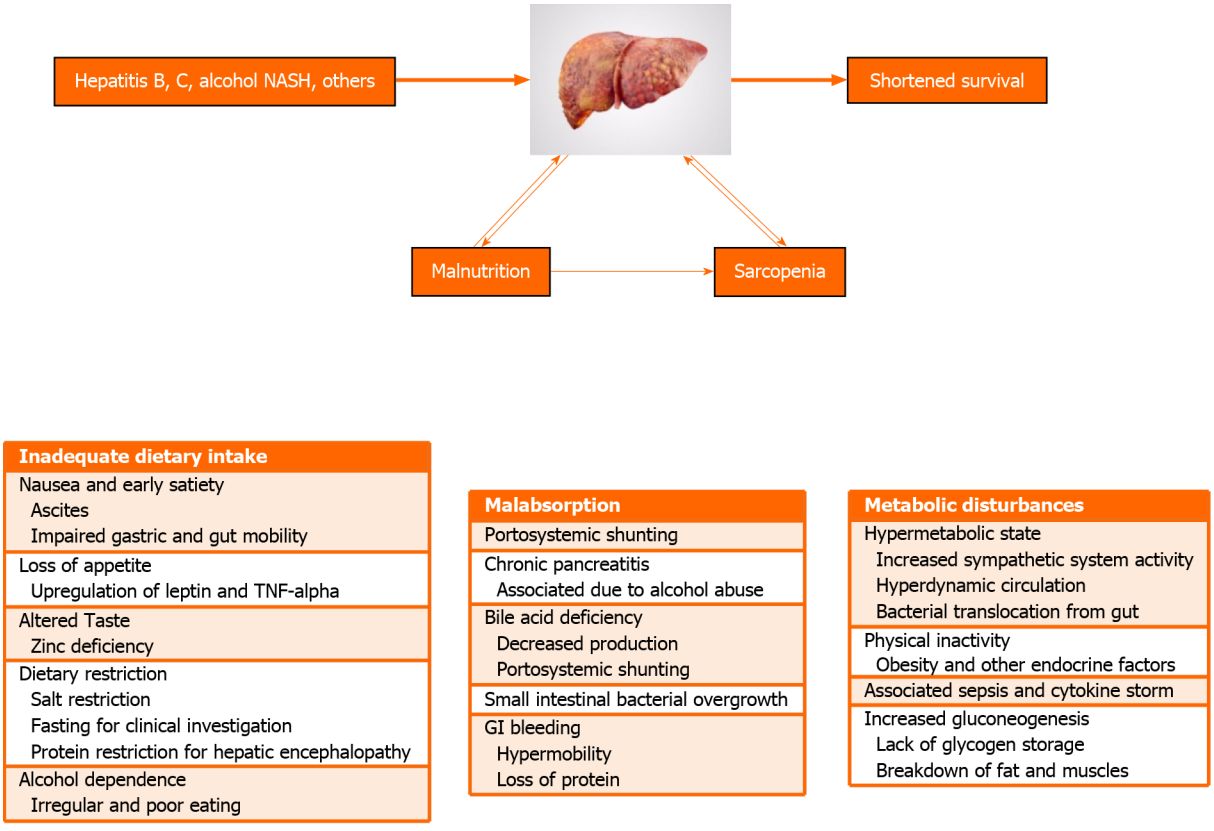


Figure 2 Pathophysiology of malnutrition as a consequence of cirrhosis and the multiple pathways that lead to it. GI: Gastrointestinal; NASH: Nonalcoholic steatohepatitis; TNF: Tumor necrosis factor.

have been diagnosed with type 2 diabetes mellitus due to glucose intolerance or end stream resistance to insulin seen in an estimated 70% of cirrhotic patients[29]. It has also been observed that the levels of hepatic and muscle glycogen are decreased, leading to a reduction in the availability of glucose as an energy substrate, with the body switching to the consumption of fats and proteins as alternative energy sources [30].

Regarding the rest energy expenditure (REE), a measure of resting metabolic rate and defined as the energy expended to sustain homeostasis at rest, conflicting results have been reported. Up to 34% of patients have been claimed to have a REE out of range by being about 120% higher, while some results show most of the patients with a REE within range[9,31,32]. Further measuring the calorie consumption, a cross-sectional study of 473 patients with cirrhosis found that 34% had hypermetabolism, measured by indirect calorimetry. The increase in REE was associated with lean body mass and an increase in beta-adrenergic activity but not with the severity or type of liver disease. Further studies have demonstrated that hypermetabolism can persist at least 1 year after liver transplant and correlates with a reduction in survival, also indicating prognosis[33]. Ascites can also contribute to an increase in REE due to which its removal results in a significant reduction in the REE when measured by indirect calorimetry. The mechanism underlying this observation is unclear[34].

The muscle plays a significant role in metabolism particularly of amino acids (AA). It also constitutes an important site for glutamine synthesis and gluconeogenesis. It should be noted that gluconeogenesis plays a direct role in the destruction of muscle tissue, with the body deriving protein substrates from the muscle for metabolism. This destruction of muscle subsequently causes a decrease in the synthesis of proteins and in its break down, also causing these patients to become protein deficient. Therefore, the most common complication of cirrhosis is a presentation of sarcopenia, observable in almost 60% of patients[35,36]. The ratio of branched chain AA (BCAA) to aromatic AA declines in CLD. A similar effect is seen in sepsis and major trauma. It is due to this change that malnourished individuals often develop hepatic encephalopathy (HE). This has established sarcopenia as an important risk factor for HE[36,37]. Further contributing to the increase in the consumption of AA by the muscles is the factor of any overnight fast that promotes an increase in ketogenesis and gluconeogenesis, even with lipids representing 75% of the calories expended during such a period. Healthy subjects after 3 d of fasting may also have a similar effect, but it occurs much slower when compared with cirrhotic patients[38].

There is an overall loss of protein from reduced synthesis of urea and hepatic proteins, reduced intestinal protein absorption and increased urinary nitrogen excretion[39]. The deficiency of AA and thus proteins gives rise to specifically a protein-calorie malnutrition (PCM). Each stage of CLD is affected by PCM with PCM being present in 65%-90% of patients with advanced disease. PCM also detrimentally causes systemic complications. There is an increased risk of HE and hepatorenal syndrome, while esophageal varices may often be seen as well. There is also a reduced regeneration capacity of the liver with a declining trend of liver function and an increased risk of surgical morbidity as well as mortality, all due to the protein and AA deficiency causing subsequent metabolic dysfunction[40,41].

Peripheral insulin resistance and β-cell dysfunction due to abnormal cell signaling pathways in cirrhotic patients leads to pancreatic dysfunction, giving rise to hepatogenous diabetes, steatorrhea and overall pancreatic disease[42,43]. The improper carbohydrate metabolism preferentially causes lipids to be oxidized instead for energy. This increases lipolysis and fatty acid oxidation, while an increase in the production of ketones may also occur. Therefore, reduced levels of plasma triglycerides, phospholipids, cholesterol, apoproteins and polyunsaturated fatty acids occurs in cirrhotic patients over time. These decreasing levels can be correlated with the severity of the liver disease, becoming an independent predictor of mortality in alcoholic cirrhosis. The respiratory quotient is also lowered when compared to non-diseased patients, also pointing towards poorer patient outcomes[44,45].

Deficiency of micronutrients

Micronutrient deficiency is common in cirrhosis, most observed in alcoholic disease. The most common deficiency seen is that of group B vitamins. Deficient levels of micronutrients are explained mainly by a decreased oral intake, malabsorption and declining hepatic reserves[46]. Fat-soluble vitamins such as Vitamin A, D, E and K have been seen to be deficient in alcoholic disease, along with steatorrhea, cholestasis and bile salt deficiency, negatively impacting patient outcomes[12,47]. Thiamine deficiency is also often found particularly in alcoholic disease as well as other forms of cirrhosis. Intake of alcohol causes a decline in the intestinal absorption and metabolization of thiamine. This increases the risk of developing Wernicke encephalopathy and Korsakoff dementia due to a decline in neurotransmitter synthesis, nucleic acid synthesis and synthesis of steroids and fatty acids[48]. Decreasing liver reserves cause a vitamin B12 and folic acid deficiency. While the levels of vitamin B12 in the serum may be increased, decreased tissue levels are observed. Anemia, glossitis and neurological symptoms may subsequently occur[12,49].

Retinol deficiency also occurs in cirrhosis and is related with decreased absorption and impaired hepatic mobilization due to hepatic stellate cell injury. This may result in dermatitis, night blindness, dyslipidemia or photophobia and increase the risk of hepatocellular carcinoma and other neoplastic disorders[50-52]. Vitamin A supplementation should be administered carefully because its excess causes hepatotoxicity [53]. Vitamin K deficiency results from declining hepatic reserves, increasing the risk of bleeding; while vitamin D deficiency can be caused from a reduced ingestion and absorption. Absorption is impaired in underexposure to ultraviolet light as well as in cholestatic disease or portal hypertension enteropathy[54]. Patients with abnormal liver functioning have deficient vitamin D levels as hydroxylation is required to synthesis calcidiol. A significant part of the liver loses function before calcidiol production is seen to be decreased. It is due to this that patient presentation with biochemical or histological evidence of osteomalacia is rare, unless a vitamin D deficiency is present concomitantly[55,56]. Therefore, decreasing vitamin D levels directly increase the risk of patient mortality where CLD is also present. It also contributes to failure of treatment of liver diseases especially in patients being treated for hepatitis C [57].

Other deficiencies present in patients with alcoholic and nonalcoholic liver disease include that of zinc and selenium. Their supplementation has improved CLD outcomes[58]. Zinc deficiency is caused by a reduction in intake and systemic assimilation. Treatment with diuretics is also a contributor. The levels of ammonia rise in the circulation, which causes dysregulation of hepatocyte functioning and an increased risk of HE. Anorexia can also be caused with this deficiency, along with the dysfunction of the immune system[38,59]. Magnesium deficiency is also noted with alcohol consumption causing not just folate deficiency but also contributing to decreasing magnesium levels. Magnesium deficiency was observed in 30% to 80% of alcoholics. Dysfunctional magnesium transport and homeostasis in vital organs such as the brain, skeletal muscle, heart and liver can result due to alcohol intake. Often magnesuria can also occur[60]. The subsequent magnesium deficiency can cause dysgeusia and contribute to minimal HE, which further decreases the patients' intakes and worsens outcomes. Clinical improvement is therefore noted with magnesium supplementation [54]. Table 1 summarizes the effects of each micronutrient deficiency caused by liver disease.

NUTRITIONAL ASSESSMENT IN CLD

A nutritional assessment determines both the macro and micronutrient state of a patient. It is necessary to identify nutritional risks that affect morbidity and mortality and helps develop a targeted therapy to benefit the patient.

Malnutrition is more evident in decompensated compared to compensated cirrhosis. The 2019 European Association for the Study of the Liver guidelines recommend a screening of all advanced CLD patients for malnutrition (undernutrition), as it is a recognized complication of liver cirrhosis. Criteria for patients at high risk of malnutrition are: (1) a BMI < 18.5 kg/m^2; and (2) advanced decompensated cirrhosis (Child-Pugh C patients)[57].

Thus, an assessment and diagnosis of malnutrition should be done thoroughly starting with a detailed history and physical examination. A thorough history can help identify poor intake, eating habits, loss of appetite, weight changes and any episodes of diarrhea and malabsorption as a potential source of malnutrition in a patient. Calculation of the patient's BMI and determination of the Child Pugh Score for severity of disease is also important with the history, as a direct correlation exists between severity of disease and malnutrition. A physical examination also provides insight about any macro and micronutrient deficiencies. Specific physical findings, such as pallor in iron deficiency anemia, are very important to observe and record[9, 61].

Skeletal muscle loss (sarcopenia) has been established as the most common complication of cirrhosis[59]. It is associated with severe outcomes in patients with liver cirrhosis and is considered useful for the global assessment of patients with CLD, therefore a physical examination for it is important[62]. A multitude of different standardized nutritional status assessment tools also exist that can be used for examination.

Subjective global assessment scale

This includes components of history and physical exam and does not rely on any lab

Table 1 Effects of each micronutrient deficiency caused by liver disease

Micronutrient	Effect caused by deficiency in liver disease
Vitamin A	Dermatitis, night blindness, dyslipidemia, photophobia, increased risk of neoplasia
Vitamin B1	Wernicke encephalopathy and Korsakoff dementia
Vitamin B9	Dementia
Vitamin B12	Anemia, glossitis and neurological symptoms (numbness, muscle weakness and ataxia)
Vitamin D	Rare osteomalacia, increased risk of mortality
Vitamin E	Reduced antioxidation
Vitamin K	Increased risk of bleeding
Zinc	Hepatocyte dysfunction, increased risk of hepatic encephalopathy
Magnesium	Dysgeusia, increased risk of hepatic encephalopathy

parameters[63]. Weight changes, dietary intake changes, presence of gastrointestinal symptoms, functional capacity and the metabolic demand associated with the disease state are the historical components, while the physical examination components include the presence of edema, ascites, muscle wasting and subcutaneous fat loss. The scale classifies patients from well-nourished to severely malnourished[64]. However, this scale underestimates the presence of sarcopenia and is not a good outcomes predictor[65].

Royal free hospital subjective global assessment

This is a modified version of the subjective global assessment and includes some objective variables as well like anthropometry and gender of the patient[66].

Assessment of sarcopenia

Computed tomography scan: Computed tomography scan can be utilized to calculate the cross-sectional area of muscles at the L3 vertebral level. The psoas, paraspinal and abdominal wall muscles are used to calculate their skeletal muscle area. The skeletal muscle index is calculated by dividing the skeletal muscle area by the patient height. A value of the skeletal muscle index less than 50 for men and less than 39 for women is considered to meet the criteria for sarcopenia. Computed tomography scan is an expensive tool and not routinely performed but if done for screening of hepatocellular carcinoma, evaluation for liver transplant or vascular shunt can be utilized for assessment of sarcopenia and calculation of the skeletal muscle index[67,68].

Anthropometry: Anthropometry as a screening tool for nutritional assessment can prove to be important in resource limited settings. The easiest and least technically challenging is the measure of the BMI using anthropometric measurements. However, the use of BMI as a nutritional assessment tool in patients with advanced liver disease is limited as patients are often in a volume overload state, which affects their body weight[9]. A corrected BMI can be calculated instead after paracentesis of ascitic fluid or by using dry body weight[65,69]. Mid arm muscle circumference is an important alternative measurement and is not reflective of the overall body volume status. Mid arm muscle circumference shows good correlation with body cell mass measurement, which is a proven marker for PCM in CLD patients. Mid arm muscle circumference is related to mortality risk in CLD patients and hence holds prognostic value as well[70]. Skin fold thickness measurement can also be used as a nutritional status assessment tool. Thickness can be measured at the triceps, biceps, subscapular and supra-iliac areas and is representative of fat reserve. It recognizes malnutrition earlier than the BMI[61].

Dual-energy X-ray absorptiometry scan: Dual-energy X-ray absorptiometry is a radiological modality that is used to measure bone mass, fat mass and fat free mass, which is representative of lean muscle mass[71]. Its use is limited in cirrhotic patients as fat free mass can also include water mass.

Bioelectrical impedance analysis

Bioelectrical impedance analysis is a low cost, easy to administer screening tool. Alternating current is applied using two electrodes, and the impedance recorded. This

can be performed at the bedside. Bioelectrical impedance analysis is used to estimate total body water, fat mass and hence fat free mass. However, bioelectrical impedance analysis was shown to underestimate malnutrition status as compared to the PCM score in a study from Pakistan. PCM score is thus calculated using anthropometric measurements and some lab parameters[40].

Liver specific tools

A few specific nutrition screening tools have been developed for liver disease patients and these include the Royal Free Hospital-Nutrition Prioritizing Tool and the Liver Disease Undernutrition Screening Tool. The Royal Free Hospital-Nutrition Prioritizing Tool score includes components involving weight loss, volume overload, BMI and reduced oral intake. Patients are then classified as being at low, moderate or high risk for malnutrition[72]. The Liver Disease Undernutrition Screening Tool includes components of oral intake, weight loss, loss of subcutaneous fat or muscle mass, volume overload and functional status[73,74]. These tools have not been studied in detail in the South Asian Population.

Dietary assessment

A dietary interview with the patient provides important insight to the patient's nutritional status. It recognizes barriers to nutritional intake and any deficiencies that need to be addressed. A thorough evaluation includes number of meals eaten, time between each meal, protein and fluid intake and total calories consumed as well as any nausea, vomiting, diarrhea or constipation. A thorough evaluation takes time and requires trained staff. Additionally, patient factors such as poor recall and lack of cooperation also play a role. A 3 d food diary is the best method for a dietary assessment, but it requires patient cooperation as well as patients being capable of writing and maintaining, which may be difficult in the mostly uneducated population of South Asia. Therefore, repeated short-term 24 h recalls are recommended and may prove to be important in this population[75,76]. Patients should at least be asked if their food intake has changed and by how much over what period of time.

In a study evaluating reasons for food aversion in a Pakistani population, it was concluded that factors such as advice from family doctor, close friends and relatives and alternative medicine practices influenced dietary restrictions in patients with advanced liver disease[77]. Lack of awareness about proper dietary intake was prevalent in both educated and uneducated populations with CLD in this population[78]. Table 2 summarizes the methods of nutritional assessment.

TREATMENT

General caloric and protein intake

A multidisciplinary approach for the treatment of malnutrition in liver cirrhosis is important for improving patient survival and long-term outcomes[79]. Nutritional counseling should be included in the patient management. According to the 2020 European Society for Clinical Nutrition and Metabolism guidelines, patients of CLD and a sedentary lifestyle should consume a daily calorie intake of 1.3 × REE (Resting Energy Expenditure)[80], where indirect calorimetry has been used to calculate the REE.

Patients with cirrhosis are recommended to consume 3 to 5 meals per day ensuring there are short starvation periods in between. A late evening carbohydrate snack has been shown to improve protein metabolism in cirrhosis by affecting nitrogen balance [81-83]. Patients should have an energy intake of 30-35 kcal/kg per day and a protein intake of 1.2-1.5 g/kg per day, whereas non-malnourished patients are recommended to intake 1.2g/kg per day of protein, and malnourished and sarcopenic patients should consume 1.5 g/kg per day of protein. However, a higher caloric intake is recommended for patients with acute decompensation or conditions with increased energy expenditure. REE should be calculated whenever possible[80]. In patients with HE, there is no recommendation for protein restriction[84-86].

Use of BCAA

In patients who are protein intolerant (develop HE with ingestion of mixed proteins), consumption of BCAA (at the rate of 0.25 g/kg per day) or vegetable derived proteins is recommended[80,87].

Table 2 Nutritional assessment methods for chronic liver disease

Nutritional assessment methods	Description
Subjective global assessment (SGA)	Uses components of history and physical exam
Royal free hospital subjective global assessment (RFH-SGA)	Modified version of SGA, includes anthropometry and gender
Sarcopenia assessment	
Computed tomography scan	Used to calculate skeletal muscle area and the skeletal muscle index at the L3 vertebral level
Anthropometry	Mid arm muscle circumference, skin fold thickness, BMI calculation
Dual-energy X-ray absorptiometry	A radiological modality that is used to measure bone mass, fat mass and fat free mass
Bioelectrical impedance analysis	Alternating current is used to estimate total body water, fat mass and fat free mass
Liver specific tools	
Royal Free Hospital-Nutrition Prioritizing Tool	Includes weight loss, volume overload, BMI, and reduced oral intake, classifies patients according to risk of malnutrition
Liver Disease Undernutrition Screening Tool (LDUST)	The LDUST includes components of oral intake, weight loss, loss of subcutaneous fat or muscle mass, volume overload, and functional status
Dietary assessment	Includes dietary review, 3 d food diary, 24 h diet recall

BMI: Body mass index; SGA: Subjective global assessment.

Prescription of long-term BCAA supplement is recommended for patients with advance cirrhosis. Use of BCAA enriched formula has been shown to improve survival in patients with alcoholic steatohepatitis and cirrhosis with an improved mental state in protein intolerant cirrhotic patients with HE[6,88-91]. However, the cost of BCAA supplements needs to be considered when prescribing them to patients.

Micronutrients

In cirrhotic patients, any micronutrient deficiency should be corrected. Micronutrient deficiencies may include both water soluble (especially thiamine) and lipid soluble deficiencies[92,93]. While there are no proven therapeutic effects directly by correcting micronutrient deficiencies, supplementation of zinc and vitamin A may improve dysgeusia and hence nutritional intake and affect the nutritional status indirectly[93,94]. While zinc deficiency has been associated with HE, supplementation has not shown any significant therapeutic effect on the encephalopathy[95-99]. Practically, a liberal supplementation is recommended in the first 2 wk of therapy, as a diagnosis of a deficiency would be costly and take time[80].

If a low sodium diet is prescribed in the scenario of ascites, the increased risk of lower intake should be considered due to the non-palatability of such a diet[100,101]. Ensuring adequate nutrition intake is important with sodium restriction.

Enteral and parenteral nutrition

If patients are unable to tolerate diet orally or if the nutritional target is unmet with oral nutrition, enteral nutrition (EN) is recommended. An adequate nutrient intake is the primary goal in cirrhotic patients. EN has been proven to be beneficial in survival and liver function[84,89]. However, with EN a risk of overfeeding exists, as the energy intake can exceed the recommended intake.

If the patient is unable to maintain oral and EN, parenteral nutrition (PN) should be used. When administering parenteral nutrition, patients are more prone to infection and sepsis through intravenous lines[25].

CONCLUSION

Malnutrition in liver cirrhosis is a serious problem in South Asia where the etiology differs from the Western population. As malnutrition is generally highly prevalent in the region, it causes an impact on patients with liver cirrhosis. Urgent attention to address malnutrition is needed to improve patient outcomes. Emphasis on assessment for nutritional status at the initial visit with recording of vital signs is needed.

Simultaneously, treating physicians need to be made aware of the misconceptions surrounding nutritional restrictions in cirrhosis so that patient education is done correctly based on proper scientific evidence.

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REVIEW

Brain-gut-liver interactions across the spectrum of insulin resistance in metabolic fatty liver disease

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Abstract

Metabolic associated fatty liver disease (MAFLD), formerly named "nonalcoholic fatty liver disease" occurs in about one-third of the general population of developed countries worldwide and behaves as a major morbidity and mortality risk factor for major causes of death, such as cardio-vascular, digestive, metabolic, neoplastic and neuro-degenerative diseases. However, progression of MAFLD and its associated systemic complications occur almost invariably in patients who experience the additional burden of intrahepatic and/or systemic inflammation, which acts as disease accelerator. Our review is focused on the new knowledge about the brain-gut-liver axis in the context of metabolic dysregulations associated with fatty liver, where insulin resistance has been assumed to play an important role. Special emphasis has been given to digital imaging studies and in particular to positron emission tomography, as it represents a unique opportunity for the noninvasive in vivo study of tissue metabolism. An exhaustive revision of targeted animal models is also provided in order to clarify what the available preclinical evidence suggests for the causal interactions between fatty liver, dysregulated endogenous glucose production and insulin resistance.

Key Words: Metabolic associated fatty liver disease; Nonalcoholic fatty liver disease; Endogenous glucose production; Insulin resistance; Steatohepatitis; Inflammation

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Core Tip: From studies using tissue-targeted animal models, it emerges that neither insulin resistance per se induces hepatic steatosis, nor steatosis induces whole-body insulin resistance. However, it is evident that reducing inflammation has several beneficial effects both at the hepatic and whole-body level. In fact, either hepatic or systemic inflammation act as major throttle of progressive liver and systemic diseases.

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INTRODUCTION

In recent years, nonalcoholic fatty liver disease (NAFLD) has become the leading cause of chronic liver disease worldwide, and the endpoint complication of nonalcoholic steatohepatitis (NASH), a major indication for liver transplantation[1]. The magnitude of the problem is highlighted by a recent model that estimated a 178% increase in deaths caused by liver disease related to NASH by 2030[2]. Among noncirrhotic NAFLD patients, the leading cause of death is cardiovascular disease[3]. Fatty liver associates with the metabolic syndrome and predisposes to all diseases (cardiovascular, digestive, metabolic, neoplastic and neurodegenerative) that are major causes of death in developed countries. As metabolic dysfunctions play a major role in the pathogenesis of fatty liver, a panel of experts has recently proposed to change the term "NAFLD," and its definition, to metabolic associated fatty liver disease (MAFLD)[4,5] in an attempt to identify clinical criteria to give a "positive" diagnosis of the disease. Furthermore, the definition of "nonalcoholic" was misleading because it is virtually impossible to exclude the endogenous production of alcohol by an intestinal autobrewery[6]. According to the new consensus, MAFLD means the evidence of hepatic steatosis accompanied either by type 2 diabetes (T2D) or overweight/obese or in normal weight/lean subjects by at least two metabolic risk abnormalities.

There are many complex physio-pathologic connections within the brain, gut, and liver (BGL) axis (Figure 1). While MAFLD per se contributes to an increased risk of neurodegeneration[7,8], one well known alteration of this axis is linked to hepatic encephalopathy (HE) a debilitating neuropsychiatric condition often associated with acute liver failure and/or cirrhosis[9]. However, the pathophysiologic mechanisms and treatment options involved in HE are much different from those involved with insulin resistance in MAFLD. Consistently, a recent study demonstrated that the nonabsorbable antibiotic rifaximin, a standard of care for HE had no effect on improving insulin resistance, adipose tissue inflammation, or plasma lipopolysaccharide (LPS) levels following an oral lipid test in obese subjects[10].

Our review is focused on the early pathophysiology of BGL in the context of insulin resistance and specifically addresses two pillars of hepatic insulin resistance, namely dysregulated endogenous glucose production and MAFLD. Of note, highly selected patients with MAFLD without any features of the metabolic syndrome, have altered endogenous glucose production[11]. Specific emphasis will be given to novel findings from imaging studies, since imaging provides noninvasive in vivo "snapshots" of the tissues of interest. We and others have highlighted that there is an imperative need of noninvasive techniques, including imaging to identify effective biomarkers and early prognostic patterns of MAFLD[12,13]. Finally, we discuss new insights that can be gained from targeted animal models in which interventions such as the knockout (KO) of the insulin receptor or the GLUT4 glucose transporter in different tissues, or the primary upregulation of lipid synthesis help to elucidate the effect of insulin resistance on hepatic steatosis and *vice versa*.

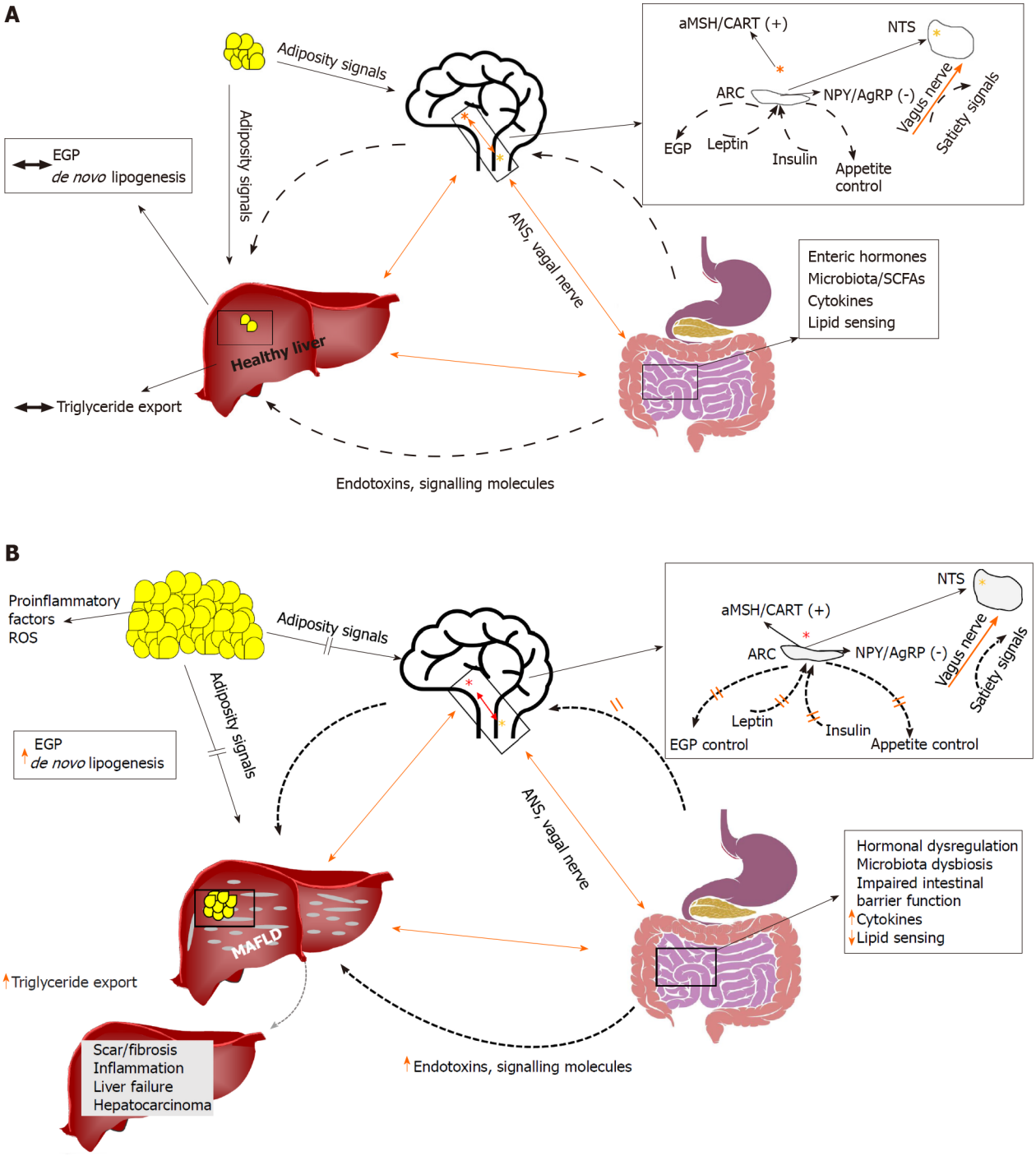


Figure 1 A summary of some interactions (A) of the brain, liver, and gut in health, and (B) in the context of insulin resistance. Several lines of research have shown that the brain may directly control endogenous glucose production. Recent evidence suggests that the brain may also control the rate of lipid turnover in the liver, thus promoting or defending from metabolic associated fatty liver disease (MAFLD). The liver is anatomically in close relationship to the gut, which represents the first line of defense against gut-derived endotoxins and signaling molecules (*e.g.*, short-chain fatty acids). Altered gut microbiota and/or a leaky gut may contribute directly to establishment of MAFLD. The gut also produces substantial amounts of hormones that, through endocrine signals, act on the brain and the liver. The autonomic nervous system and the vagus nerve constitute the basis of the brain, gut and liver axis interconnections. Orange line: liver–brain–gut neural arc; dotted line: other ways of communication (*e.g.*, hormonal, adipocytokines). Red star denotes the hypothalamic nuclei; yellow star denotes the nucleus tractus solitaries. αMSH: α-melanocyte-stimulating hormone; AgRP: Agouti-related protein; ANS: Autonomous nervous system; ARC: Arcuate nucleus; CART: Cocaine- and amphetamine-regulated transcript; EGP: Endogenous glucose production; MAFLD: Metabolic associated fatty liver disease; NPY: Neuropeptide Y; NTS: Nucleus tractus solitarius; ROS: Reactive oxygen species; SCF: Short-chain fatty acid.

MAFLD-INSULIN RESISTANCE-INFLAMMATION: A VICIOUS CIRCLE

Whereas the association between MAFLD and insulin resistance is well established, there is debate on their cause-effect relationships. Thus, it is not clear whether systemic insulin resistance induces the accumulation of lipids in the liver[11] or if hepatic

steatosis is a major determinant of systemic insulin resistance[14]. In any case, once hepatic steatosis is established, other typical characteristics are observed such as insulin resistance, insufficient suppression of endogenous glucose production (EGP), increased insulin secretion, decreased whole-body glucose disposal, increased lipolysis with consequent enhanced lipid oxidation[11], decreased insulin clearance[15], and chronic oxidative stress[16]. Both insulin resistance and MAFLD are characterized by elevated circulating inflammatory markers[17,18]. The interplay between insulin resistance, ectopic fat accumulation in the liver, and inflammation is characterized by mutual positive regulation, *i.e.* a vicious circle[19]. On one hand, ectopic fat accumulation in the liver leads to lipotoxicity, low-grade inflammation and insulin resistance in the liver[19]. On the other hand, insulin resistance enhances lipotoxicity through unsuppressed lipolysis[19]. Finally, proinflammatory markers such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, which are typically increased in conditions of insulin resistance may further aggravate both insulin resistance and MAFLD[19].

In MAFLD patients, intrahepatic inflammation is the most important prognostic determinant of liver disease progression and systemic inflammatory markers correlate with hepatic inflammation[20]. A plausible hypothesis holds that in the context of MAFLD, inflammation (hepatic and/or systemic) acts as major disease accelerator[13].

BRAIN-LIVER AND BLG AXIS

Control of EGP

Preclinical studies have shown that insulin acting directly on the brain may affect EGP. More specifically, Obici *et al*[21] have shown that an intracerebroventricular (ICV) injection of insulin suppresses EGP in rats. Human evidence is slowly accumulating, and recent clinical studies have confirmed the presence of a "brain-liver axis". More specifically, intranasal insulin (INI) administration during the euglycemic hyperinsulinemic clamp was shown to suppress EGP in lean, but not in overweight, individuals[22]. Under the same euglycemic hyper-insulinemic conditions, brain imaging with ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography (PET) has shown that brain glucose uptake (BGU) correlates positively with EGP in morbidly obese individuals, but not in healthy lean individuals[23]. On the contrary, when INI was given during fasting conditions EGP was not affected, and similarly no correlation was found between BGU and EGP in the post-absorptive state. Taken together, the data suggest that under conditions of high systemic insulin levels like those typically seen in the postprandial state, the brain may directly control (*i.e.* suppress) EGP, but the control is lost with increased adiposity.

Other lines of research also suggest that the brain may control EGP. Intracerebroventricular administration of brain-derived neurotrophic factor (BDNF) lowers blood glucose levels and suppresses hepatic glucose production (HGP) and hyperglucagonemia[24]. Of note, leptin activates BDNF-expressing hypothalamic neurons, which in turn stimulate BDNF synthesis[25]. Also, Bercik *et al*[26] have shown that the intestinal microbiota affects both the central levels of BDNF and behavior in mice.

Lipid delivery into the upper intestine has also been shown to suppress EGP through central action. More specifically, lipid delivery leads to long chain fatty acyl-CoA production that suppresses EGP, and the effect is abolished either after coadministration of the anesthetic tetracaine, by gut vagal deafferentation, or by hepatic vagotomy[27]. Wang and colleagues further demonstrated that in rats with insulin resistance induced by a high-fat diet (HFD), upper intestine lipid delivery failed to suppress EGP, suggesting a potential mechanism for dysregulated EGP in the context of insulin resistance. Along the same line, cholecystokinin (CCK) has also been reported to trigger gut-brain-liver axis control of HGP, and that HFD impaired CCK-induced afferent vagal signals to suppress HGP[28].

Enteric hormones (enteroendocrine system)

The gastrointestinal tract is a major producer of hormones, among which GLP-1, ghrelin, cholecystokinin are particularly involved in the BGL axis. GLP-1, an incretin hormone secreted from the L cells of the intestine in response to a meal, exhibits high fasting levels in subjects with insulin resistance and in whom GLP-1 does not increase sufficiently in response to a meal[29]. GLP-1-receptor mRNA has been found both in the hepatic portal region and in neurons[30]. Preclinical studies have suggested that GLP-1 may act on the liver through the nerve endings of the intestinal wall. Insulin clamp studies in GLP-1 receptor knockout mice showed a defective suppression of EGP[31], and the intraportal GLP-1 injection in rats increased the firing rate of the

hepatic afferent of the vagus nerve[32]. In healthy individuals under conditions of pancreatic clamping (*i.e.* with stable insulin and glucagon concentrations), GLP-1 inhibits EGP, and the effect is mediated either through direct GLP-1 action on the liver or through neuron-mediated inhibition[33]. GLP-1 also contributes to suppression of appetite[34]. Thus, GLP-1 may play an important role in the pathophysiology of MAFLD; and in preliminary clinical studies, exenatide, a GLP-1 receptor agonist, was shown to decrease hepatic fat content and liver enzymes[35], and to improve liver histology[36]. Similarly, liraglutide was shown to improve NASH[37].

Ghrelin is a hormone that is mainly derived from the stomach and duodenum, and its main function is to control food intake by inducing appetite. Ghrelin has recently been shown to participate in the BGL axis, as under conditions of pancreatic clamping, intraduodenal ghrelin infusion resulted in increased HGP through neural-mediated action, as administration of ghrelin, while inhibiting the vagal afferent neurotransmission, abolished the ghrelin-induced increase of HGP[38]. Similarly, vagotomy or use of N-Methyl-D-aspartate blockers abolished the ghrelin effects on HGP. Ghrelin has also been shown to block the action of cholecystokinin. The ghrelin nutrient-sensitive effects on the gut may thus be attributed to its inhibition of cholecystokinin.

CCK is released from intestinal endocrine cells during feeding, and it binds to CCK-A receptors on gut vagal fibers that project the signal to the brainstem, causing termination of the meal[39]. Insulin and CCK have complimentary actions in inducing satiety, as ICV administration of insulin enhances the satiety effect of CCK[40], whereas fasting decreases it[41]. Similar complementary effects with CCK have been proposed for leptin, as ob/ob mice and Zucker rats have been shown to be relatively insensitive to the satiety effects of CCK[42].

Adipocytokine signaling

Leptin is an adipokine, or adipocytokine with structural similarities with the cytokines of the type I cytokine family. Circulating leptin levels are directly related to expanded fat mass, but obesity is characterized by leptin resistance, and leptin resistance consists at least partially in a decreased capacity for leptin transport into the brain[43]. Even though secreted by adipose tissue, the main site of action of leptin is the central nervous system (CNS), and particularly in the hypothalamic nuclei. Leptin and insulin central actions are largely interconnected; both act on the arcuate nucleus to suppress the expression of the orexigenic peptides neuropeptide Y and agouti-related protein. Their action on other neurons is different and more complex, as leptin stimulates while insulin inhibits proopiomelanocortin neurons[44]. Apart from controlling EGP, appetite (and thus body weight), leptin is also implicated in MAFLD as it has been demonstrated that leptin deficient ob/ob mice have marked steatosis[45]. A recent preclinical study has shown that CNS-leptin signaling promotes hepatic triglyceride export and decreases de novo lipogenesis[46]; the authors propose intranasal leptin administration as potential new treatment of MAFLD.

Vagus nerve and the enteric nervous system

As already highlighted, the tenth cranial, or vagus nerve, plays a pivotal role in the BGL axis communications, which are summarized in Figure 1. The enteric nervous system, also named the "second brain" or "brain in the gut" is considered as one of the autonomic nervous system divisions and consists of approximately 500 million neurons which produce a variety of neurotransmitters including acetylcholine, adrenaline, VIP and serotonin (5-HT). It has been shown that 5-HT promotes lipid accumulation in hepatocytes in vitro[47]. In line with that, short-term treatment with tryptophan inhibitors prevented the formation of 5-HT, which is a metabolite of tryptophan, and inhibited the development of hepatic steatosis in mice fed with a high carbohydrate diet without increasing the energy expenditure in adipose tissues[48]. In addition, the same study showed that inhibition of gut-derived serotonin ameliorated hepatic steatosis. Taken together, the data suggest that gut-derived serotonin is a regulator of hepatic lipid metabolism through a gut-liver axis. On the other hand, the gut microbiota regulates both the 5-HT synthesis and its release from the enteroendocrine cells, and 5-HT plays its role on the CNS as one of the most important central neurotransmitters in the regulation of mood, sleep, and pain[49]. Consistently, modification of central 5-HT levels was shown by Pagoto *et al*[50] to affect food preferences. Following acute tryptophan depletion (transient decrease of both peripheral and central 5-HT levels), overweight individuals increased their sweet calorie intake and preferred sweet foods. Of significant note is that most tryptophan was converted to kynurenine rather than to 5-HT, and under conditions of inflammation, the rate-limiting enzyme for this transformation could be upregulated. As kynurenine and 5-HT compete to cross the blood-brain barrier through the same

transporter, it follows that inflammation-associated changes in kynurenine levels could impact on central 5-HT concentrations[51,52]. Thus, this is another pathway through which inflammation could decrease central 5-HT levels to prompt affected patients to increase their intake of sweets.

Gut microbiota

An altered gut microbiota composition is associated with obesity[53], and in obese humans specific microbiota compositions may be associated with impaired glucose control[54]. It is well established that a diet rich in fibers is healthy, with improvement of insulin sensitivity and glucose tolerance. The beneficial effects of increased fiber consumption are hypothesized to be mediated by the production of the short-chain fatty acids (SCFAs) acetate, propionate, and butyrate after fermentation of the fibers by the gut microbiota. SCFAs do not act just as substrate for colonocytes and enterocytes [55], but also as signaling molecules. For instance, SCFAs can stimulate the secretion of GLP-1 and peptide YY, and decrease the secretion of ghrelin[56-58]. Propionate and butyrate have been shown to activate intestinal gluconeogenesis (IGN), and interestingly, increased IGN was shown to have beneficial effects on glucose homeostasis even if the resulting increase in EGP is a key feature of T2D[59]. In the case of propionate, the effect occurs *via* a gut-brain neural circuit[60], as it was shown that after denervation of the periportal nervous system, propionate feeding no longer affected IGN. Even though butyrate feeding (which has a direct effect on IGN shown by gene expression *via* a cAMP-dependent mechanism) could still enhance IGN, both the beneficial effects of IGN and portal glucose sensing were lost[60].

Decreased levels of the SCFA butyrate have also been associated with tight-junction abnormalities and increased intestinal permeability[61], which have been implicated in MAFLD pathogenesis and progression. Other microbiota-derived molecules might play also an important role. Deficiency of the macronutrient choline, which is implicated in the prevention of liver steatosis by promoting the assembly and excretion of very-low-density lipoprotein[62], has been observed in NAFLD patients and was associated with abundance of specific bacteria (Erysipelotrichia taxa), which are able to metabolize choline to trimethylamine (TMA) and its oxidized form TMAO, with the net effect of reducing choline bioavailability and increasing that of steatogenic TMAO[63].

Amino acid metabolism is also important. The branched-chain amino acids (BCAAs) valine, isoleucine and leucine, contribute to insulin resistance and hepatic steatosis, and can be synthesized and metabolized by specific gut bacteria. An intervention study in rats showed that the dietary administration of BCAA reduced the accumulation of liver fat through the modification of gut microbiota[64]. The effect occurred through the gut-brain axis, accompanied by microbiota-mediated production of the SCFA acetate, which activated the parasympathetic nervous system[65]. Other amino acids, such as tryptophan, phenylalanine, and tyrosine can be metabolized by gut bacteria that produce derivates with effects on metabolism and inflammation. For example, the essential amino acid tryptophan is the precursor of serotonin and can be converted into its indole intermediate, which in turn can reduce hepatic lipogenesis and inflammation[66].

Furthermore, the bacterial-derived endotoxin lipopolysaccharide (LPS) might contribute to local and systemic inflammation by the activation of the toll-like receptor 4 pathway. Increased abundance of endotoxin-producing bacterial strains has been found in the gut of obese patients compared with controls[67], suggesting its potential implication in the development of MAFLD and its progression to NASH, with the involvement of CNS dysfunction and inflammation.

Dietary patterns are able to modulate brain lipid composition and function[68] as well as hepatic lipid content[69]. It has been shown that unhealthy diets rich in saturated or monounsaturated fatty acids have unfavorable effects on gut microbiota composition[70], promoting an increase of LPS-producing bacteria and reduction of SCFAs leading to a systemic proinflammatory state occurring through the BGL axis [71].

Finally, gut microbiota-dependent regulation of neurotransmitters interacts with vagal afferent pathways to affect liver metabolism through the gut-brain axis. Gut bacteria can modify serotonin release[72], which has a brain-dependent effect on gastrointestinal motility and secretion, and energy expenditure. It promotes liver steatosis by local endocrine mechanisms[73], and modulates the production of several other molecules, such as gamma-aminobutyric acid, acetylcholine, histamine, norepinephrine, dopamine, and endocannabinoids that affect glucose and lipid metabolism and inflammation, as deeply reviewed elsewhere[74].

A useful experimental model for studying the effects of specific microbiota on host metabolism is provided by germ-free mice, which are resistant to high-fat diet-induced obesity[75] and protected from MAFLD. In a seminal study Le Roy *et al*[76] showed that germ-free mice that received fecal transplantation from C57BL/6J mice with HFD-induced hyperglycemia and increased plasma concentrations of proinflammatory cytokines, developed hepatic steatosis. On the contrary, germ-free mice that received stool from C57BL/6J mice that were non-responders to HFD and without hyperglycemia and increased proinflammatory cytokines, did not develop hepatic steatosis. Moreover, the gut microbiota controlling the balance between proinflammatory and anti-inflammatory signals may contribute to the progression to NASH. Consistently, short-term treatment of patients with steatosis and NASH with rifaximin led to an improvement of liver enzymes levels[77]. The effect was thought to have been caused by a change of the gut microbiota composition leading to a direct reduction of leaky gut, and consequent improvement of hepatic inflammation, rather than an effect on insulin sensitivity. It was recently demonstrated by Finlin *et al*[10] that rifaximin did not improve insulin resistance.

Bile acids

Bile acids (BAs) are synthesized in the liver from cholesterol, stored in the gallbladder, and secreted after gallbladder emptying into the intestinal lumen upon food ingestion. As BAs move along the intestinal lumen, they contribute to the absorption of lipids and lipophilic vitamins. The majority of BAs (~95%) are re-absorbed by enterocytes and then transferred back to the liver where they are reused (*i.e.* enterohepatic circulation). BA are transformed to secondary BAs by the gut microbiota[78] and only in a small amount reach the systemic circulation and increases of plasma BA levels were reported after meals, suggesting that BAs could be a postprandial systemic signal [79].

In the last decades, important new insights have been gained, proposing BAs as important determinants of glucose homeostasis. Early studies showed that KO of the BA farnesoid X receptor (FXR) induced insulin resistance, whereas administration of BA agonists enhanced insulin sensitivity[80]. BA receptors are also present in the CNS, and it is now believed that the BA signal reaches the brain through three different pathways, one direct and two indirect[81]. The direct pathway consists in activating central FXR and Takeda G protein-coupled receptor (TGR5R) signaling after crossing the blood-brain barrier. Indirect activation of intestinal FXR and TGR5R results in the release of FGF19 and GLP-1, both of which can signal to the CNS. The pathways have been extensively reviewed by Mertens *et al*[81]. Even though major pathways of communication of the BGL axis through BAs were identified, their importance in pathophysiology warrants further investigation.

Nutrient intake: Role of fructose

In Western societies, increased consumption of fructose began in the 1970s after the introduction of high-fructose corn syrup as a sweetener in soft drinks. Since then, the prevalence of obesity and T2D have substantially increased and a link between high fructose consumption and MAFLD has been established[82]. Fructose is a 5-carbon carbohydrate with peculiar characteristics, as upon entry in the cell it is phosphorylated to fructose-1-phosphate by phosphofructokinase, decreasing the cell's ATP levels because of the rapid depletion of phosphate. AMP degradation increases uric acid levels, which has a proinflammatory affect in the intracellular compartment. In the liver, fructose can be transformed into free fatty acids that can either be secreted into the circulation as triglycerides or stored as intrahepatic lipids, contributing to MAFLD. How does fructose affect the liver through the BGL axis? Studies in mice have shown that fructose consumption causes a strong binge-eating response that is attributed to release of orexin from the lateral hypothalamus[83]. Chronic fructose intake leads to leptin resistance and weight gain[84]. Similar findings were confirmed in humans, who after fructose assumption, experienced increased hunger and desire for sweet foods than after glucose administration[85]. Furthermore, Spruss *et al*[86] showed that the long-term intake of fructose was associated with a marked reduction of the protein in the tight junctions of the duodenum that led to an increase in translocation of bacterial endotoxin and activation of toll-receptor-4-dependent signaling cascades in the liver. Interestingly, metformin a drug that reduces insulin resistance, was shown to protect from fructose-induced steatosis[86].

RESULTS OBTAINED FROM TISSUE-TARGETED ANIMAL MODELS

Based on the frequent coexistence of fatty liver, steatohepatitis, obesity, T2D, dysbiosis, insulin resistance, and low-grade inflammation, there is general acceptance of their possible causal interactions. However, their exact nature and implication in different subgroups of patients remains to be elucidated. In order to identify more specific causal relationships, we reviewed studies in tissue-targeted animal models in which the known primary event was either insulin resistance or hepatic steatosis or gut microbiota depletion or the induction or reduction of inflammation (Figures 2 and 3; Figure 2 is given more extensively in Supplementary Table 1). All the studies indicate that unless extreme lipodystrophy occurs (FIRKO-90%), severe insulin resistance in the whole body or skeletal muscle and/or adipose tissue does not cause hepatic steatosis and liver insulin resistance or inflammation[87-89]. GLUT4-null mice do not have hepatic steatosis or glucose intolerance and have normal EGP-related enzyme expression in their liver[90-92]. In the absence of insulin receptors or GLUT4 in both muscle and adipose tissue, glucose tolerance is normal or is less affected than expected from the degree of insulin resistance[93-95]. The key compensatory organs appeared to be adipose tissue (with upregulation in glucose uptake, increase in small adipocyte number), and the liver (with upregulated glucose uptake, balanced increase in lipid synthesis *vs* export). A chronic lack of insulin action in only the liver leads to unsuppressed EGP, resulting in severe glucose intolerance from early life onward, low insulin clearance, glycogen depletion, low expression of lipogenesis pathways, and resistance to high-fat diet-induced steatosis[96-99]. The effects of a short-term lack of insulin action are controversial[98,100]. With a normal diet in the chronic model, moderate liver steatosis occurs in older animals, with an elevation in liver enzymes, focal dysplasia, no fibrosis, and low circulating triglycerides, which may depend on blunted triglyceride export because of chronic brain exposure to hyperinsulinemia. In fact, acute *vs* chronic central insulin infusions have shown a transition from stimulation to suppression of hepatic triglyceride export[101], modulating liver fat content. Lack of insulin action in the brain also causes a moderate degree of hyperphagia in females (either with or without overweight), insulin resistance without hyperglycemia, or glucose intolerance and high or normal triglyceride levels[101-103]. Instead, the selective KO of brain GLUT4 results in normal peripheral insulin sensitivity, but unsuppressed EGP leading to glucose intolerance[104]. Although the intestine is assumed to contribute little to EGP, it was noted that the absence of insulin action in enterocytes ameliorated glucose tolerance, *via* reduced intestinal glucose absorption and downregulation of intestinal EGP enzyme expression[105,106]. From these studies, EGP (dys)regulation resulting from the action of insulin on the liver, brain, and gut seems to be the most prominent determinant of glucose (in)tolerance.

On the other hand, the primary induction of liver steatosis does not cause whole-body insulin resistance, glucose intolerance, or hepatic inflammation, but provokes an increase in hepatic ceramide and diacylglycerol content, and the enrichment of liver triglycerides with polyunsaturated fatty acids, which may increase susceptibility to inflammatory damage[107-111]. The exposure to toxins (including LPS) caused liver and lipid inflammation and reduced fasting glucose, insulin and triglyceride levels [108-111]. Consistently, older studies have shown that an injection of LPS leads to a major increase in fasting glucose consumption by the whole body, with a several-fold elevation in the liver and spleen glucose uptake lasting 48 h, possibly because of the content of macrophages[112]. More recent studies on chronic LPS infusion have revealed that, while inducing overweight and inflammation in the liver and in adipose tissue and muscle in chow and HFD fed mice, LPS caused steatosis and extra-hepatic insulin resistance only in HFD mice or hepatic insulin resistance (EGP) only in chow-fed mice, with a small impact on glucose tolerance[113]. This is in agreement with observations in germ-free mice lacking LPS showing lower liver fat, better glucose tolerance, higher insulin sensitivity, and normal circulating triglyceride and free fatty acid levels compared with colonized mice[114-116]. However, the selective inoculation of bacteria producing or not producing LPS in germ-free mice showed a direct effect only on adipose tissue inflammation and without hepatic or systemic impact[116]. All the evidence suggests that both the metabolic and hepatic effects of LPS require other microbial or dietary components and support a role for liver inflammation, but not steatosis per se, in the regulation of peripheral metabolism. In line with this, anti-inflammatory drugs have been shown to ameliorate liver function, steatosis, inflammation, and insulin resistance, with glucose and lipid lowering effects observed only in diabetic animals[117-121].

Primary defect	Species	Targeted model	Liver Steatosis	Liver	EGP during clamp	Whole body IR-IS	Fasting TG-FFA	Fastingglucose	Glucose intolerance	Body weight	Adiposity	Reference
Peripheral insulin resistance	Mouse	MIRKO										Bruning Mol Cell 1998, Kim J Clin Invest 2000, Ealey Am J Physiol 2008
	Mouse	FIRKO 50%										Bluher DevCell 2002, Boucher Diabetes 2016, Softic Diabetes 2016
		FIRKO 90%										
	Mouse	GLUT4-KO-M (muscle), GLUT4-KO-M+AT (muscle + adipose tissue)										Carvalho AJP 2005, Zisman Nat Med 2000, Kim JCI 2001, Kotani JCI 2004
	Mouse	GLUT4-null										Lin and Accili J Biol Chem 2011, Ranalletta Diabetes 2005 and AJP 2007, Katz Nature 1995
Insulin resistance in liver, brain, intestine	Mouse	LIRKO										Michael Mol Cell 2000, Fisher & Kahn JCI 2003, Buettner JCI 2005, Coen J Biol Chem 2007, Biddinger Cell Metab 2008, Haas Cell Metab 2012
	Mouse	LGSKO										Irimia J Biol Chem 2010, 2017
	Mouse	NIRKO (or ins. Infusions, or WB ins. Receptor deletion or sparing brain)										Bruning Science 2000, Diggs-Andrews Diabetes 2010, Scherer Diabetes 2016
	Mouse	Brain specific GLUT4 KO + chow or HFD										Reno Diabetes 2017
	Mouse	Intestinal-IRKO										Andres Am J Physiol 2014, Ussar Diabetes 2017
Hepatic steatosis ± inflammation	Mouse	Hepatic DGAT2 or MTTP overxpression										Monetti Cell Metab 2007, Minehira J Lip Res 2008 Raabe JCI 1999, Bjorkegren J Biol Chem 2002, Jornayvaz PNAS 2011
	Mouse	MTTP overexpr + LPS or ConA or PEA										Bjorkegren J Biol Chem 2002
Microbiota ± inflammation	Mouse	Gut microbiota KO (Germ-free)										Rabot FASEB J 2010, Bäckhed PNAS 2007, Caesar Gut 2012
	Mouse, rat	LPS, effects of microbiota in chow diet (in HFD normal EGP, peripheral IR, liver steatosis)										Meszaros J Biol Chem 1987, Cani Diabetes 2007, Caesar Gut 2012
Inflammation-targeting	Mouse, rat	NSAID (cox-inhib, aspirin, indomethacin, IL1Ra) effect in high-risk models (diet, BDL, TAA, LDLR-KO, GK)										Madrigal-Perez Int J Clin Exp Med 2015, Paik Gut 2009, Tian Plos One 2014, Murali J Lip Res 2012, Ehses PNAS 2009

Figure 2 Tissue-targeted animal models evaluating the independent effects of tissue-specific insulin receptor knockout, or GLUT4 knockout, or the induction of steatosis or inflammation on metabolic outcomes, including liver steatosis, endogenous glucose production, glucose tolerance, and body weight. Negative effects are shown in red and abnormalities reported as mild-moderate or not consistent in all studies in light red. Beneficial effects (dark) or no effect (light) are shown in green. Orange indicates opposite (high *vs* low) findings between studies. Light blue refers to a decrease that cannot be unequivocally interpreted as being beneficial or adverse to health. BDL: Bile duct ligation; ConA: Concanavalin A; DGAT2: Diacylglycerol O-acyltransferase 2; FFA: Free fatty acids; FIRKO: Fat-specific insulin receptor knockout; GK: Goto-Kakizaki; HFD: High-fat diet; IL-Ra: Interleukin-1 receptor antagonist; IR: Insulin resistance; IRKO: Insulin receptor knockout; KO: Knockout; LDLR-KO: Low-density lipoprotein cholesterol receptor knockout; LIRKO: Liver-

specific insulin receptor knockout; LGSKO: Liver glycogen synthase knockout; LPS: Lipopolysaccharide; MIRKO: Muscle-specific insulin receptor knockout; MTTP: Microsomal triglyceride transfer protein; NIRKO: Brain-specific deletion of the insulin receptor; PEA: P. aeruginosa exotoxin A; TAA: Thioacetamide; TG: Triglycerides

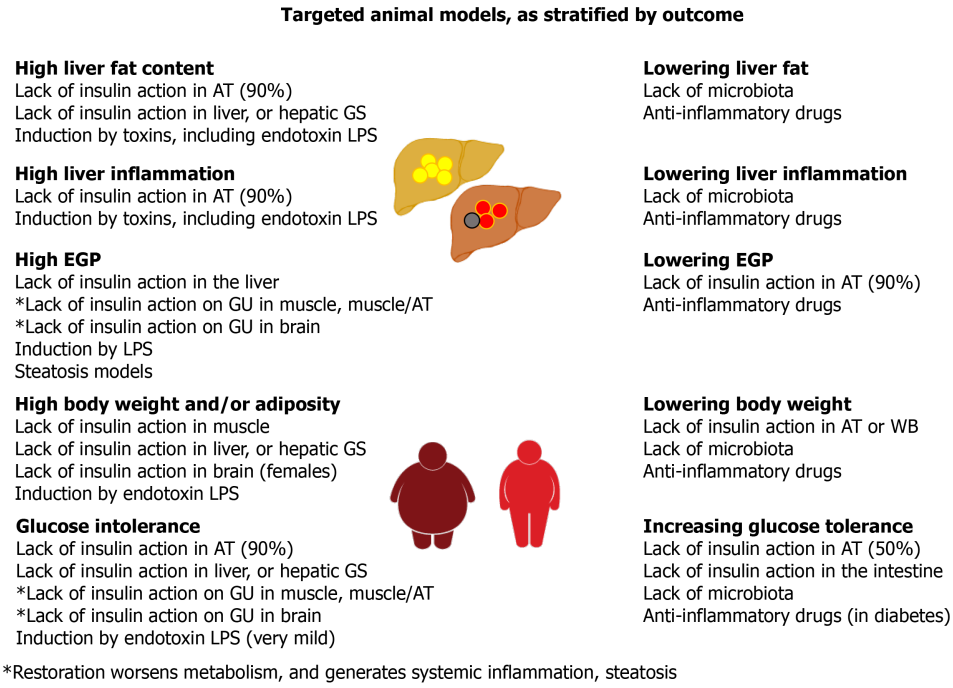


Figure 3 Summary of the outcomes yielded by targeted animal models. AT: Adipose tissue; LPS: Lipopolysaccharide; GS: Glycogen synthase; GU: Glucose uptake; WB: Whole body.

DISEASE MONITORING BY DIGITAL IMAGING: FOCUS ON PET AND MECHANISTIC UNDERSTANDING

Liver biopsy is the current gold standard in both the diagnosis and follow-up of liver disease. The presence of ballooning degeneration of hepatocytes being the hallmark of steatohepatitis, but biopsy is invasive and unsuitable for frequent monitoring[122]. Liver function tests are useful, but not diagnostic or predictive of NASH and/or fibrosis in individual patient. Imaging tools can capture and measure liver steatosis. Among them, magnetic resonance imaging (MRI)-magnetic resonance spectroscopy (MRS) has the highest sensitivity, but it is complex and not always accessible. Recently mono- and multiparametric scores obtained by the AI-processing of common ultrasound images have been proposed for repeated follow-up of liver fat and are validated against spectroscopic magnetic resonance technology[123]. Vibration-controlled transient elastography and magnetic resonance elastography provide useful measures of the combined inflammation-fibrosis index, but a reliable distinction between them remains to be achieved in the diagnostic field[12].

MRI can measure the proton density fat fraction (PDFF) and has been shown to be an objective, accurate, and reproducible quantitative indicator of hepatic fat content across the entire liver. MRI-PDFF has been validated against liver histology, and shown to be more sensitive in detecting changes in hepatic fat content and treatment response in clinical trials[124-126].

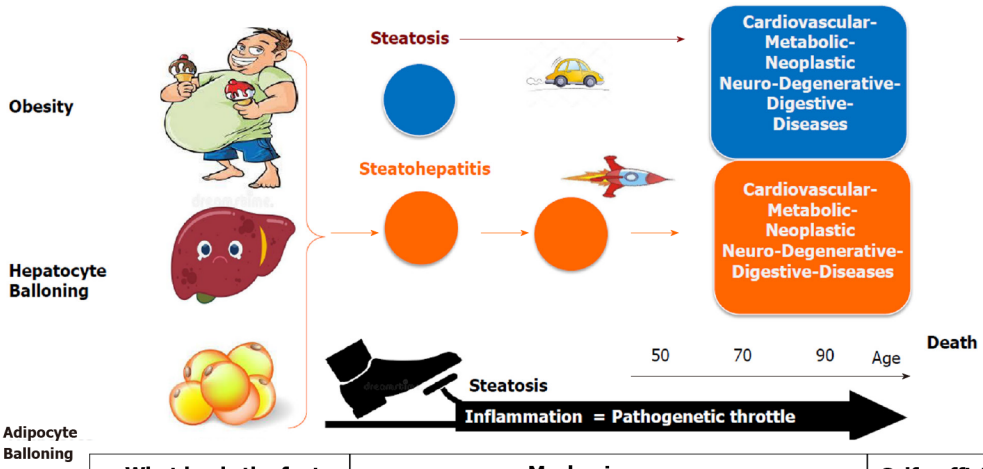
Finally, multiparametric MRI makes it possible to establish scores for assessment and quantification of liver fibrosis and inflammation, with accurate prediction of clinical outcomes in patients with chronic liver disease of mixed etiologies and/or steatosis[127]. The animal studies discussed above showed that LPS-induced liver inflammation is characterized by very high hepatic glucose uptake, possibly because of macrophages. The notion that activated macrophages and lymphocytes have high glucose-avidity has supported the use studies with PET imaging of the glucose analogue (^{18}F)-FDG in inflammatory conditions, such as osteomyelitis, sarcoidosis, vasculitis, or vulnerable atherosclerosis plaques[128,129]. Some studies, although lacking liver biopsies, have explored the relationship between computed tomography-

determined steatosis and fasting (^{18}F)-FDG-PET imaging[130-132], yielding controversial results. Two recent reports that used biopsy-proven liver diagnosis and compartmental modeling of (^{18}F)-FDG in the liver, found an inverse relationship between hepatic inflammation grades and liver blood flow, *i.e.* the K_1 rate constant representing the flow-dependent delivery of (^{18}F)-FDG to the liver[133,134]. Other rate constants (*e.g.*, fractional extraction) did not correlate with histology grades. Unfortunately, these human studies addressed relative indices and not the absolute rate of hepatic glucose uptake (HGU), which is given by the product of (^{18}F)-FDG fractional uptake × circulating glucose levels, and they did not quantify EGP.

We have previously validated a method to simultaneously estimate EGP [by (^{18}F)-FDG plasma clearance] together with HGU (by imaging) during (^{18}F)-FDG-PET, addressing their relationship with liver steatosis (by MRI-MRS) in type 2 diabetic or morbidly obese patients. The studies indicate that hepatic insulin resistance and steatosis are, to some extent, proportional and improve after weight loss by bariatric surgery in morbidly obese individuals[135]. However, very-low-calorie diets in less severe obesity had effects on glucose tolerance, EGP, and liver fat, but not on HGU [136], whereas glucose lowering by SGTL2 inhibitors in diabetic patients had a significant effect on glucose control and liver fat, but not on EGP or HGU[137]. Taken together, the studies suggest that liver fat is not a cause of hepatic dysmetabolism, but rather a consequence of glucose intolerance. It is also important to keep in mind that the euglycemic insulin clamp that was used in the studies, did not reflect the daily metabolic physiology of patients, in which glucose and insulin levels increase and decrease together after meals or under fasting conditions. HGU and EGP are dependent on the changing insulin and glucose levels, and chronic hyperglycemia and hyperinsulinemia are commonly present. For example, PET imaging studies in minipigs underscore the relevance of circulating glucose by showing that hyperglycemic- compared with euglycemic-hyperinsulinemia enhanced HGU, hepatic triglyceride content and triglyceride release in proportion to glycemia[138]. The euglycemic clamp thus provides relevant information on the sole action of insulin on tissue metabolism, being insufficient to characterize the more complex relationship between glucose and lipid metabolism occurring in the liver in real life.

By using a fatty acid PET tracer, we demonstrated that overweight was characterized by an elevation in fasting hepatic fatty acid oxidation, with normal rates of triglyceride incorporation[139]. Liver steatosis occurred in obese subjects, in whom weight loss was able to reduce hepatic fatty acid uptake and liver steatosis in a proportional manner, and EGP[140]. Notably, a chronic, *i.e.* 1-wk treatment with acipimox, suppressing fatty acid levels and liver fatty acid uptake provoked a significant improvement in systemic and liver insulin sensitivity and decreased circulating triglycerides and liver enzymes, but did not change liver fat content, as measured with MRS in healthy individuals[140]. Thus, liver and systemic insulin sensitivity were improved, together with liver function and independent of hepatic triglyceride accumulation. Again, in spite of cross-sectional correlations and consensual changes after weight loss, intervention studies disconnect liver steatosis per se from other adverse metabolic consequences, at least in healthy subjects.

In spite of the new light on pathophysiology shed by the above studies, two major needs remain unmet. Firstly, none of the above PET imaging studies included sufficient histologic information to address the progression of liver steatosis into steatohepatitis and/or fibrosis, thus the specific factors of disease progression are not yet identified. However, sufficient knowledge exists to design targeted studies for a more effective demonstration of the potential of PET-CT as diagnostic tools. Secondly, the relevance of other organs in compensating or aggravating liver disease needs a better understanding in order to address appropriate treatment strategies and targets, and intervention-time windows. We have just started to examine the brain-liver-gut axis in humans by PET imaging. Our studies during euglycemic clamp revealed a positive relationship between BGU and EGP and the predictive value of BGU of glucose homeostasis in diabetic subjects following bariatric surgery[23]. We also detected a high fasting-uptake of fatty acids in the brain in obese and morbidly obese individuals[141]. A greater elevation of BGU was also observed in reward-related but not in behavior-controlling regions in response to sensory stimulation by chocolate stimuli in overweight women with high food-addiction scores, compared with women with lower scores, independent of peripheral substrate and hormone levels, which were shown to be similar. Only in the former group was BGU reduced after a low-calorie diet, independent of similar peripheral changes[142]. Thus, high or unbalanced BGU is associated with a variety of high-risk behavioral and metabolic aspects. More important, the study underscores that the same phenotype can result from different mechanisms, and that mechanistic or intervention studies pooling patients based on a



What loads the foot	Mechanisms	Self-sufficient
Extreme deficiency in adipose tissue substrate storage	Build up of reactive oxygen species in the steatotic liver, lipid peroxidation, macrophage infiltration, later dysplasia	Yes
Hepatic triglyceride composition	Unsaturated fatty acids render the steatotic liver more susceptible to (endo/exo) toxin-mediated oxidative damage, lipid peroxidation	No
Unestablished gut microbiota factors	Germ-free (GF) conditions trigger protective responses	Yes
Lipopolysaccharide (LPS)	Hepatic proinflammatory factor, but not self-sufficient (proinflammatory effect lost in GF conditions)	No

Figure 4 Threesome ballooning hallmark of progressive fatty liver disease. Liver steatosis is associated with cardiovascular, digestive, metabolic, neoplastic, and neurodegenerative diseases; but it is the concomitant presence of inflammation that markedly accelerates the progression of these diseases. We illustrate this concept, representing steatosis as a "pedal" on which a series of aggravating factors may press as a "foot" that pushes on the "throttle", namely inflammation.

similar phenotype (*e.g.*, obesity or T2D) may be misleading both on detection of cause and on the evaluation of treatment efficacy. Evaluating the BGL axis by PET imaging with double-tracer oral glucose loading, we showed that the administration of exenatide (a GLP-1R agonist) in subjects with impaired glucose tolerance decreased EGP and HGU. A decrease in the intestinal absorption of oral glucose resulted in lower insulin levels, with an increased proportion of orally ingested glucose that was retained by the liver and increased BGU in most brain regions[143]. That underlines the importance of integrating intestinal metabolism and absorptive effects under real life circumstances in the study of the BGL axis. The quantification of intestinal glucose uptake by PET imaging has been recently validated, showing that intestinal insulin resistance in the jejunum was improved by bariatric surgery in obese subjects and in the large and small intestine by metformin and mildly improved in the small intestine of diabetic patients by rosiglitazone. Intestinal fatty acid uptake was elevated and further increased in obese subjects after bariatric surgery. Interestingly, parallel animal model observations showed that the human body could release glucose and fatty acids from the circulation into the gut lumen[144], which suggests that the gut can be a way to actively eliminate excess substrate and that the body feeds substrates to the gut microbiota, potentially modulating its composition and function. EGP was either decreased, unchanged or increased in the studies, again indicating a possible confounding effect of morbid obesity or a disconnect between insulin action in gut and liver. These studies, primarily planned to address insulin sensitivity, have set the stage for the design of gut-targeted and BGL-targeted imaging approaches under metabolic conditions that are relevant to this interaction, including the study of microbiomics.

CONCLUSION

We reviewed the most recent knowledge of the complex interplay among the organs of the BGL axis in the pathophysiology of insulin resistance and MAFLD, presenting the best established interconnections between brain, gut and liver in the context of insulin resistance and hepatic steatosis. From studies using tissue-targeted animal models it

emerges that insulin resistance per se does not induce hepatic steatosis, nor does steatosis induce whole-body insulin resistance. However, it is evident that reducing inflammation has several beneficial effects both at the hepatic and whole-body level. In fact, inflammation (either hepatic or systemic) acts as major throttle of progressive liver and systemic diseases. This paradigm is illustrated in Figure 4 together with the important diagnostic and prognostic role of the three hallmark characteristics of progressive fatty liver disease, namely visceral obesity (abdominal ballooning) and/or ballooning degeneration of hepatocytes and adipocytes (thus predominant adipocyte hypertrophy rather than hyperplasia).

There is currently no approved treatment for MAFLD, which is a multifaceted syndrome caused by pathogenetic mechanisms that, in animal studies, consistently appear to be diverse. Understanding and being able to identify and measure different factors that trigger and/or accelerate the pathogenesis of steatosis and its progression is a key issue for successful risk-stratification, prevention, and drug development.

With several drugs being potentially beneficial in the treatment of MAFLD, future clinical investigations should address carefully the most appropriate stratification of MAFLD patients to study their specific effects on liver and systemic inflammation, liver fat, and insulin resistance. On the other hand, the approach of both system biology and medicine has to be applied to address the unmet needs in the understanding of the pathophysiology of insulin resistance in different subsets of patients with MAFLD. The pooling of MAFLD patients just on the basis of their common phenotypic characteristic in clinical studies and trials can be highly misleading for both mechanistic understanding and for new drug development as the same phenotype can result from different and time/stage-evolving mechanisms, each requiring a very targeted approach (*i.e.* personalized, timely, and adequate for the disease-stage). Studies targeting the BGL axis might unveil new underlying mechanisms and fill the existing knowledge gaps in the causal links between insulin resistance and MAFLD pathophysiology, paving the way for the development of innovative diagnostic and therapeutic approaches.

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REVIEW

Gut microbiome in acute pancreatitis: A review based on current literature

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Abstract

The gut microbiome is a complex microbial community, recognized for its potential role in physiology, health, and disease. The available evidence supports the role of gut dysbiosis in pancreatic disorders, including acute pancreatitis (AP). In AP, the presence of gut barrier damage resulting in increased mucosal permeability may lead to translocation of intestinal bacteria, necrosis of pancreatic and peripancreatic tissue, and infection, often accompanied by multiple organ dysfunction syndrome. Preserving gut microbial homeostasis may reduce the systemic effects of AP. A growing body of evidence suggests the possible involvement of the gut microbiome in various pancreatic diseases, including AP. This review discusses the possible role of the gut microbiome in AP. It highlights AP treatment and supplementation with prebiotics, synbiotics, and probiotics to maintain gastrointestinal microbial balance and effectively reduce hospitalization, morbidity and mortality in an early phase. It also addresses novel therapeutic areas in the gut microbiome, personalized treatment, and provides a roadmap of

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human microbial contributions to AP that have potential clinical benefit.

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Core Tip: We live in a world of microbes. There is a distinct microbiome sighted in every niche of our body. This review is based on current knowledge to define an overview of how the gut microbiota has accelerated the frontiers of understanding recently and empowered its importance in influencing human physiology through its potential role in various diseases. It further explores the possible application of microbiota-targeted therapeutics in routine clinical practice, meaning manipulating gut microbiota into the current therapeutics to minimize the potential risk of various diseases, including acute pancreatitis.

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INTRODUCTION

Recent research has confirmed the importance of the human gut microbiota in maintaining health and its involvement in disease. The human gastrointestinal (GI) tract harbors a diverse microbial population of more than 10^{14} microorganisms, comprising more bacterial cells than human body cells[1] and more than 3.3 million unique genes[2]. The predominant commensal bacteria in the human GI tract are members of the phyla *Firmicutes* and *Bacteroidetes*, constituting 80%–90% of the total gut microbiota[3]. Other phyla include *Proteobacteria, Actinomyces, Actinobacteria, Fusobacteria,* and *Verrucomicrobia*[4]. The composition of the gut microbiome plays a key role in modulating human immune responses to invasive pathogens, and it also prevents the pathogens from crossing the intestinal barrier[5].

Recent improvement of advanced sequencing methods, such as next-generation sequencing and metagenomics, have added to our understanding of the involvement of gut microbiome in human health and disease[6] and the potential therapeutic value of interventions that target the composition of the gut microbiome. Such interventions would manipulate the host-microbiome community by eliminating harmful taxa or reconstituting missing beneficial taxa[6,7]. Recent evidence shows that, under ideal conditions, symbiotic relationships among the microbial species in the host GI tract can function to prevent opportunistic and nosocomial infections that have become more frequent because of the widespread use of antibiotics to treat various diseases[8-11].

Research performed over the past two decades has revealed the significance of gut dysbiosis in the pathogenesis of many diseases[12], including inflammatory bowel disease[13], irritable bowel syndrome[14], colon cancer[15], Alzheimer's disease[16], coronary heart disease[17], obesity[18], and diabetes mellitus[19]. Changes in the diversity, proportions, and dominant species of the gut microbiome are probably associated with intestinal barrier dysfunction that influences onset and clinical course of multiple diseases, including pancreatic disorders[20]. In a healthy individual, no gut microbes are present in the pancreas, but changes of the gut microbiota may be involved in the pathogenesis of pancreatic disease, including acute pancreatitis (AP)[21]. AP is a common disorder of the digestive system with high morbidity and mortality worldwide. Managing AP is challenging and has a heavy financial burden on the patient and society[22,23]. Therefore, it is important to understand the primary causes and mechanism of the pathogenesis and progression of AP to facilitate early diagnosis and treatment, avoid a course leading to severe disease, and reduce AP-associated fatality[23]. Ongoing studies of AP in humans have found that premature activation of trypsinogen, dysfunctional calcium signaling, impaired endoplasmic reticulum stress-related autophagy, unfolded protein response, and mitochondrial

dysfunction all promote AP. However, the cause of multiorgan dysfunction in AP is poorly understood[24]. The potential role of the intestine in promoting systemic inflammation and organ dysfunction is of interest.

In AP, hypovolemia, reflex splanchnic vasoconstriction, intestinal ischemia, and reperfusion injury due to fluid resuscitation may result in bacterial translocation [25, 26]. Systemic inflammatory response syndrome accompanied by intestinal bacterial translocation is associated with high AP mortality[26]. A change in gut permeability/motility that causes bacterial translocation and leads to the activation of gut-associated lymphoid tissues may result in systemic complications in AP[27]. This review summarizes relevant human and animal studies that provide insights into the potential role of the gut microbiome in AP pathogenesis. It also summarizes treatment perspectives that target the gut microbiome.

PATHOPHYSIOLOGY OF AP

Previous studies of microbiome involvement in AP described a complex cascade of events with significant involvement of pancreatic acinar cells, but the mechanisms involved in the initiation of AP are still poorly understood[28]. Development of a well-defined clinical management protocol is challenging. Most investigations of AP pathophysiology have documented injury or disruption of the pancreatic acini that triggered the activation of pancreatic enzymes (trypsin, chymotrypsin, and elastase) in pancreatic tissue. Activated proteases (trypsin and elastase) and lipase breakdown tissue and cell membranes, leading to edema, vascular damage, hemorrhage, and necrosis[28].

During the initial phase of pancreatic injury, acinar cells release proinflammatory cytokines, like tumor necrosis factor (commonly referred to as TNF)-α, interleukin (IL)-1 and IL-6, and anti-inflammatory mediators, such as IL-10 and IL-1 receptor antagonist[29]. These mediators recruit neutrophils and macrophages to enter the pancreatic parenchyma, to propagate both local and systemic responses. Reactive oxygen metabolites, prostaglandins, platelet-activating factor, and leukotriene may also be involved[29].

Recent epidemiological studies have shown that a local inflammatory response aggravates pancreatitis by increasing permeability, which damages the microcirculation and results in local hemorrhage and pancreatic necrosis in cases of severe AP. Some of the inflammatory mediators released by neutrophils aggravate pancreatic injury by activating pancreatic enzymes (see Figure 1)[26,30]. Figure 1 is a schematic description of AP pathogenesis. The acinar cells of the pancreas cause trypsin activation followed by impairment of cell membrane trafficking and activation of the zymogen cascade mediated by trypsin. Attraction and activation of leukocytes occur with the release of pro- and anti-inflammatory cytokines and chemokines. Overt, sustained activation of proinflammatory mediators leads to systemic inflammatory response syndrome (SIRS) and may progress to multiorgan failure, infection, pancreatic necrosis, and sepsis as late complications of AP.

CLASSIFICATION OF AP

Approximately 15%–25% of patients diagnosed with AP may progress to severe AP. The 2012 revised Atlanta classification and definitions by international consensus include three degrees of AP severity (*i.e.* mild, moderately severe, and severe)[31]. The three degrees primarily manifest as transient organ failure, persistent organ dysfunction, and local or systemic AP[31]. The determinant-based classification of AP is highly dependent on clinical data, and also on the available feedback from patients (to a lesser extent)[32]. Table 1 summarizes both the Atlanta and determinant-based classifications.

VARIOUS SCORING SYSTEMS USED IN THE DIAGNOSIS OF AP

The initial diagnosis of AP made on arrival at a clinic or hospital relies on known medical history, comprehensive physical examination, and increased serum amylase or lipase, with or without additional imaging evaluation. Currently, there are no specific laboratory tests with consistent accuracy and reliability for predicting AP

Table 1 Revised Atlanta classification and determinant-based classification of acute pancreatitis

Revised Atlanta classification of disease severity	
Mild AP	No organ failure
	No local or systemic complications
Moderately severe AP	Organ failure that resolved within 48 h (transient organ failure) and /or
	Local or systemic complications without persistent organ failure
Severe AP	Persistent organ failure > 48 h
	Single organ failure
	Multiple organ failure
A modified Marshal score defines a persistent organ failure	
Determinant-based classification of disease severity	
RACAP	**DBCAPS**
Mild AP	Mild AP
Absence of organ failure	Absence of organ failure
Absence of local complications	Absence of (peri-) pancreatic necrosis
Moderately severe AP	Moderate AP
Local complications and/or	Sterile (peri-) pancreatic necrosis and/or
Transient organ failure	Transient organ failure
Severe AP	Severe AP
Persistent organ failure	Persistent organ failure or
	Infected (peri-) pancreatic necrosis
	Critical AP persistent organ failure
	Infected (peri-) pancreatic necrosis

AP: Acute pancreatitis; DBCAPS: Determinant-based classification of acute pancreatitis severity; RACA: Revised Atlanta classification. Persistent organ failure is defined by a modified Marshal score or a sepsis-related organ failure assessment score.

severity. However, several scoring systems are available and are routinely used by hospital physicians to predict AP severity and prognosis (Table 2)[33-41].

BIOMARKERS AND PREDICTORS TO CONFIRM THE SEVERITY OF AP

In addition to the various clinical scoring systems, several biomarkers have also been applied as predictors of AP severity and are shown in Table 3[42-47].

microRNAs as biomarkers in AP diagnosis

Recently, circular microRNAs have been studied as potential diagnostic biomarkers in AP because of their specific properties, such as stability in biological fluids, simple identification, and sequence conservation among different species (Table 4)[48-60].

GUT MICROBIOME AND MICROBIOME IN AP

The human GI tract is home to a diverse and complex microbial community of bacteria, viruses, and fungi that help to maintain health and are involved in the pathogenesis of various diseases. The gut contains at least 1000 bacterial species and 100-fold more genes than have been identified in the human genome[4,61]. The microbiome is considered a hidden "metabolic organ", and it has a significant impact on well-being because of its influence on our metabolism, physiology, nutrition, and immune function[62]. It has been shown that the gut microbiome co-evolves with us;

Table 2 Prediction scoring systems used in acute pancreatitis diagnosis

No.	Multifactorial scoring system	Timeline	Threshold	Area under the curve	Ref.
1	Ranson score	48 h	≥ 3	0.81–0.88	[33-35]
2	Glasgow score	48 h	2	0.73–0.784	[36,37]
3	Acute Physiology and Chronic Health Evaluation-II score (APACHE-II)	24 h	7	0.80–0.895	[33,38,39]
4	Acute Physiology and Chronic Health Evaluation II score-Obesity (APACHE:-O)	24 h	7	0.893	[40]
5	Bedside Index of Severity score (BISAP)	24 h	≥ 3	0.79–0.875	[33-35,41]
6	Pancreatitis Activity Scoring System (PASS)	24 h	> 160	0.71	[36]
7	Systemic inflammatory response syndrome (SIRS)	24 h	≥ 2	0.73	[34,39]

Table 3 Blood biomarkers predicting disease severity in acute pancreatitis

No.	Blood biomarkers	Timeline	Threshold	Area under the curve	Ref.
1	Interleukin 8	Preoperative	196 pg/mL	0.778	[42]
2	Interleukin 6	24 h	50 pg/mL	0.9	[39]
3	Hepcidin	24 h	234.4 ng/mL	0.82	[43]
4	Red blood cell distribution width	24 h	13.35%	0.787	[44]
5	Procalcitonin	24 h	1.77 ng/mL	0.797	[45]
6	Blood urea nitrogen	24 h	5.945 mg/dL	0.677	[44]
7	Oleic acid chlorohydrin	24 h	32.40 nM	1	[46]
8	C-reactive protein	24 h	150 mg/L	0.61	[47]
9	C-reactive protein	48 h	150 mg/L	0.73–0.91	[33,39,47]

hence, any changes to the microbial community can have significant consequences, both beneficial and harmful[63]. Disruption of the gut microbiota, or dysbiosis, has been associated with diverse systematic conditions, such as obesity[64,65], malnutrition[66], diabetes[67], and chronic inflammatory diseases, such as inflammatory bowel disease, ulcerative colitis, and Crohn's disease[68].

Researchers have continued to study the microbiome at an accelerated pace over the past two decades, revealing the myriad ways these microorganisms affect our day-to-day lives. The microbiome in our gut is now understood to be a significant contributor to the development of chronic disease. Gut microbiota is now known to play a critical role in human health and disease. With the advances in microbiome research over time, more and more data has become available showing the gut microbes' overall composition and functional potential. Additionally, the number of diseases associated with changes in our gut microbial community has increased simultaneously[4,6]. The human gastrointestinal tract is a habitat crowded with microorganisms contributing to the host's immunity and pathogenesis of several diseases, including acute pancreatitis [69]. Human gastrointestinal microflora is divided into three different types by the way they present themselves in the body and perform multiple functions: *e.g.*, There are three major categories of bacteria: Physiological bacteria, that hold over 90% and are nourishing and immune-modulating; opportunistic bacteria, which are pathogenic in situations of lower immune resistance or antibiotic abuse; and pathogenic bacteria, which have lower numbers and invade difficultly[70]. Progression in AP has been more complicated by gastrointestinal motility dysfunction, which is probably related to the neuroendocrine system, hypoxia-ischemia, ischemia-reperfusion injury (IRI), inflammatory mediators, and cajal cells[71].

Since many discoveries have postulated that commensal intestinal microbiomes play a crucial role in humans' health, immune system, and homeostasis recently, there has been a surge of interest in this area of study. The overall function of the intestine in the entire mechanism of AP pathogenesis (such as in acute and critical illnesses) is essential to understand, but often it is overlooked. This pathogenesis mechanism involves several factors contributing to the loss of gut barrier function, allowing bacteria and endotoxins to translocate into the bloodstream, which is critical for

Table 4 MicroRNAs used as biomarkers in the diagnosis of acute pancreatitis

No.	miRNAs	Patients	Sample	Expression change	Reference gene	Ref.
1	miR-216a	AP	Plasma	Up	None	[49]
2	miR-551b-5p	AP	Plasma	Up	miR-16	[50]
3	miR-216a-5p, miR-375, and miR-551b-5p	AP	Serum	Up	miR-103a-3p	[51]
4	miR-7, miR-9, miR-122, and miR-141	AP	Serum	Up	Exogenous reference genes	[52]
5	miR-216a	AP	Plasma	Up	U6	[53]
6	miR-551-5p	AP	PBMC-	Up	U6	[54]
7	miR-155	AP	Serum	Up	U6	[55]
8	miR-29a	AP	Plasma	Up	U6	[56]
9	miR-24-3p, miR-222-3p, miR-361-5p, and miR-1246	HTG-AP	Serum	Up	U6	[57]
10	miR-1260b, miR-762, miR-22-3p, miR-23b, and miR-23a	AP-associated ALI	Serum	Up	U6	[58]
11	miR-92b, miR-10a, and miR-7	AP	Plasma	Down	miR-16	[50]
12	miR-155	AP	Serum	Down	Not mentioned	[59]
13	miR-181a-5p	HTG-AP	Serum	Down	U6	[57]
14	miR-550a, miR-324-5p, miR-484, miR-331-3p, miR-140-3p, miR-342-3p, and miR-150	AP-associated ALI	Serum	Down	U6	[58]
15	miR-127	AP-associated ALI	Plasma	Down	miR-16	[60]

ALI: Acute lung injury; AP: Acute pancreatitis; HTG-AP: Hypertriglyceridemic-acute pancreatitis; mi: Micro.

generating the second inflammatory hit of AP[72]. The data of Johnson *et al*[73] suggests gut bacteria translocation (*via* hematogenous, lymphatic, and reflux) is involved in AP infection progression, which indicates the presence of a possible correlation between gut microbiota and AP infection progression. An abnormality of the gastrointestinal microbiota (dysbiosis) is associated with the systematic inflammatory response syndrome (SIRS) and a broad range of diseases[74].

When the mucosal barrier of the intestine is damaged, intestinal bacteria may migrate into the blood or to other tissues and organs, further accelerating AP[75]. In recent years, several studies have been conducted investigating changes in intestinal flora associated with AP severity. AP progression involves the abnormal release of trypsin and destruction of pancreatic tissue due to abnormal cells. Several recent studies examined the changes in intestinal flora during AP development concerning disease severity. It is observed that abnormal trypsin secretion has occurred due to AP progression and that pancreatic structure destruction leads to an abnormal pancreatic secretion, resulting in the intestinal flora and homeostasis changes[76,77].

Numerous studies have now demonstrated the function of normal gut microbes to promote healthy gut mucosa. Gut mucosal ischemia and reperfusion during AP progression can compromise the integrity of the gut barrier, causing bacterial reabsorption from the gut to other parts of the body and causing local and systemic infections[78]. Some further research findings have also revealed that intestinal mucosal barrier injury is a significant complication in many AP patients. The intestinal mucosal barrier can be destroyed by affecting intestinal inflammation and the immune response[75]. Many studies are supporting now to demonstrate that normal gut microbes play a primary role in maintaining gut mucosal integrity. However, gut mucosal ischemia and reperfusion during AP progression can damage the overall integrity of the gut barrier and lead to gut bacterial translocation to other locations, causing local and systemic infections[78]. Thus, a significant complication of an AP patient's condition involves intestinal mucosal barrier damage. This is caused by intestinal inflammation and immune response defects. Other research has also found injuries of the intestinal mucosal barrier to patients with AP[75].

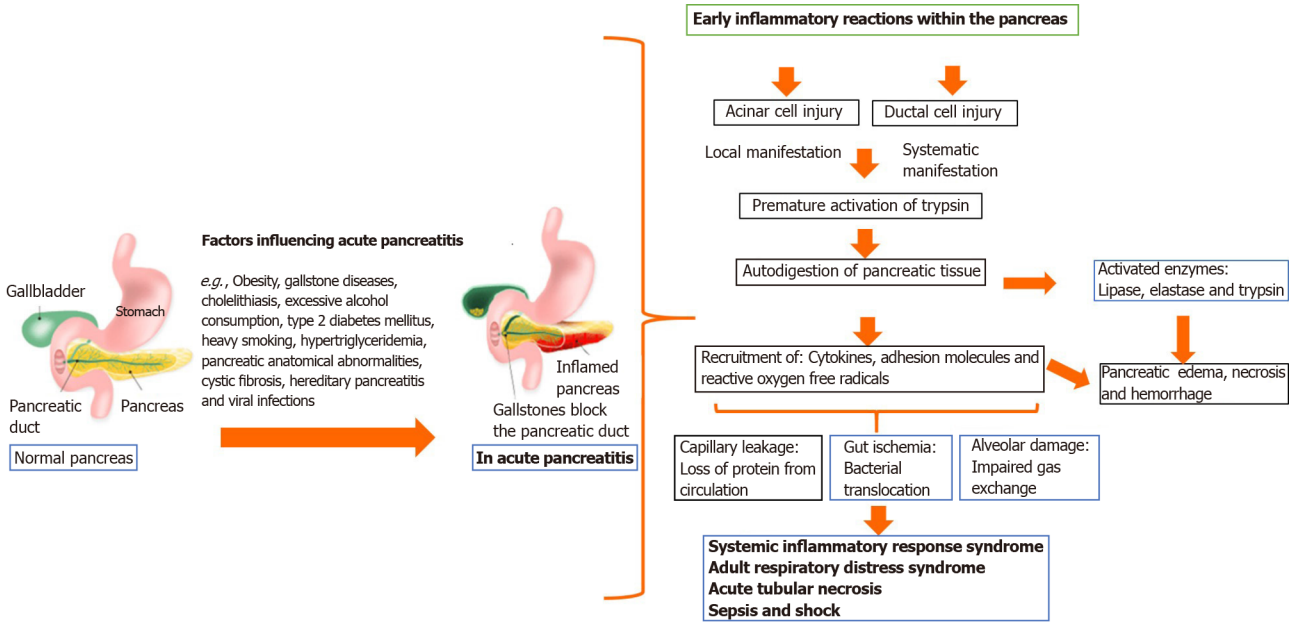


Figure 1 Pathophysiology of acute pancreatitis. Damage in the pancreas' acinar cell causes trypsin activation following cell membrane trafficking impairment, with subsequent activation of the zymogen cascade by trypsin. Attraction and activation of leukocytes occur with the release of many proinflammatory and anti-inflammatory cytokines, as well as chemokines. An overt and sustained activation of proinflammatory mediators leads to systemic inflammatory response syndrome (SIRS), which may further proceed to multiorgan failure, pancreatic necrosis and sepsis with late complications of acute pancreatitis.

In brief, the pancreas-gut communication has been described as being in AP, with bacterial translocation as a possible consequence and the homeostatic host response noted. Translocation of bacteria from the lower gastrointestinal tract occurs *via* the portal circulation - the oral course and/or the mesenteric lymph nodes. The acinar cells of the pancreas secrete pancreatic antimicrobial peptides (AMPs). AMPs have homeostatic bidirectional communication with the gastrointestinal tract[79]. The lower level of the microbiome in the gastrointestinal tract may increase pancreatic antimicrobial peptide production by short-chain fatty acid metabolites. Consequently, it induces a pancreatic immunoregulatory environment which decreases proinflammatory immune cells. Conversely, decreased antimicrobial peptide production facilitates the overgrowth of the gastrointestinal microbiota leading to the induction of proinflammatory immune cells. Thus, it subsequently alters the gut microbiome and the intestinal immune system[79].

POTENTIAL ROLE OF THE GUT MICROBIOME IN AP

Metagenomics and next-generation sequencing have facilitated the investigation of the involvement of the gut microbiome in human physiology and various diseases. The findings have allowed for consideration of the gut microbiome as a hidden organ[80]. The close interaction of the gut microbiome with host physiology can account for the harmful effects of disruptions in the former caused by various internal or external events that initiate inflammatory conditions and some types of cancer. Therefore, it is essential that specific microbial signals maintain the host immune response and other physiological functions that protect against pathogens[81].

Injury of the microcirculation and hypovolemia that occur during AP can lead to gut mucosal ischemia and reperfusion injury that result in loss of gut barrier function. Subsequent translocation of gut bacteria can result in local pancreatic and systemic infections[82]. Leading causes of AP mortality include pancreatic infection and peripancreatic necrosis[78]. The initial onset of cerulean-driven AP depends on NOD1 activation in acinar cells by commensal microbiota that have translocated from the gut. Following activation, NOD1 induces the expression of inflammatory mediators[83]. The role of the gut in neutrophil priming and release of proinflammatory cytokines is important for the initiation and propagation of inflammation and sepsis[84]. The loss of gut barrier function has been implicated in the pathogenesis of AP-related infections. Ahuja *et al*[76] reported that secretion of antimicrobials from pancreatic acinar cells regulated gut microbiota composition and innate immunity[76]. Blocking

acinar cell exocytosis in mice has been found to lead to gut dysbiosis, inflammation, systemic bacterial translocation, and ultimately, death. Additional evidence has revealed additional examples of crosstalk between pancreas acinar cells and the gut microbiome[76,77].

An ongoing investigation of the microbiota and AP has shown that microcirculatory disturbances associated with the loss of fluid into the "third space," lead to hypovolemia, ischemia, and reperfusion injury in AP patients. The gut is affected by AP, but it is not a passive victim because it plays an active role in the worsening of the illness[85]. In addition to bacterial translocation, the translocation of inflammatory compounds produced in the intestinal wall and the gut's toxic products might also be responsible for initiating SIRS and distant organ injury in AP patients[86]. The contribution of the gut microbiome for protection against pathogens in AP patients has not been clearly elucidated. An increase of such pathogenic bacteria as *Enterobacteriaceae* and *Firmicutes*, and a decrease in beneficial bacteria like *Bacteroidetes* and *Lactobacillus* has been observed in AP patients[87]. Furthermore, increased serum IL-6 has been found to be positively related to increased *Enterobacteriaceae* and *Enterococcus* and inversely related to *Bifidobacterium* and *Clostridium* cluster number. Tan *et al*[87] reported that the extent of gut microbiota modification predicted pancreatitis severity and the occurrence of systemic complications. Gerritsen *et al*[88] found that "AP-associated microbiota" replaced the normal intestinal flora in a study performed in a mouse model. In AP, changes in the populations of specific commensal bacteria have been associated with reduced levels of the inflammatory cytokines IL-1b, TNF-a, CXCL1, and IL-18, and inversely correlated with pancreatitis severity and the occurrence of systemic infectious complications. The evidence highlights the restoration of the physiological gut microbiota composition as a valuable strategy to treat AP[89].

The 16S rRNA gene is highly conserved in bacteria, and it is highly species-specific. Consequently, 16S rRNA gene sequencing is widely used to study the gut microbiota in various disease states[90]. Zhu *et al*[69] reported that the relative abundance of commensal microbiota in AP patients differed from that in a healthy individual. Members of the *Bacteroidetes* phylum decreased significantly, but *Proteobacteria* were over-represented in AP. AP patients also had a relative overabundance of *Escherichia/Shigella* compared to healthy control[69]. Increased abundance of two common opportunistic pathogens, including *Enterococcus* and an unknown genus in the *Enterobacteriaceae* family, were also observed in AP patients. Linear discrimination and effect size analysis revealed significant increases in *Acinetobacter, Stenotrophomonas,* and *Geobacillus* with decreased *Bacteroides, Alloprevotella, Blautia* and *Gemella* in patients with severe AP, as compared to those with mild and moderately severe AP[69]. Table 5[21,69,75,91] and Table 6[69,75,87,90,92] summarize the significant changes in the microbiome composition of healthy controls and in patients with mild, moderately severe, and severe AP.

Changes in the gut microbiota in AP include overexpression of opportunistic pathogens, such as *Escherichia/Shigella*, and reduced abundance of beneficial genera, such as *Bifidobacterium*[69]. It has been hypothesized that a reduction of beneficial bacteria might facilitate microbial translocation across a damaged gut barrier, thereby promoting the progression of AP. Studies performed by Li *et al*[75] and other investigators[93,94] found that dysbiosis that included the depletion of short-chain fatty acid-producing bacteria was associated with an impaired gut barrier and worsening of AP. Changes in the gut microbiome may thus serve as a diagnostic tool in AP. Restoring gut microbiota homeostasis and stabilizing the gut barrier might have therapeutic value in AP patients, as shown in Figure 2[21,30,69,75,87,93,94]. The overall literature suggests that there is an association between the gut microbiome and the severity of AP[95]. Additionally, in experimental acute pancreatitis, changes to the gut microbiome, *e.g.*, administration of *Clostridium butyricum* can suppress AP[27] pointing to the therapeutic potential of this approach is promising.

ANTIBIOTICS, PREBIOTICS, SYNBIOTICS, AND PROBIOTICS FOR THE TREATMENT OF AP

Antibiotic therapy in AP

In the past decade, substantial advancements have been made not only in understanding the pathophysiology of AP but also in treatment strategies and multidisciplinary management. Necrotizing pancreatitis occurs in about 30% of patients and has a poor prognosis and high mortality. About 80% of AP deaths are caused by infections

Table 5 Altered microbiome composition in acute pancreatitis *vs* healthy controls

No.	Techniques used for microbiome profiling	Healthy control	Acute pancreatitis	Ref.
1	qPCR (Fecal samples)	*Firmicutes*↑	*Firmicutes*↓	[84]
		Bacteroidetes↓	*Bacteroidetes*↑	
		Proteobacteria↓	*Proteobacteria*↑	
		Actinobacteria↑	*Actinobacteria*↓	
		Tenericutes↓	*Tenericutes*↑	
2	16S rRNA gene sequencing (Fecal samples)	*Proteobacteria*↓	*Bacteroidetes*↓	[83]
			Proteobacteria↑	
			Escherichia/Shigella↑	
			Enterococcus↑	
			Enterobacteriaceae↑	
			Prevotella↓	
			Faecalibacterium↓	
			Bifidobacterium↓	
3	16S rRNA gene sequencing (Fecal samples)	NA	*Bacteroidetes*↑	[85]
			Proteobacteria↑	
			Firmicutes↓	
			Actinobacteria↓	
4	16S rRNA gene sequencing (Fecal samples)	NA	*Enterobacteriaceae*↑	[21]
			Enterococcus↑	
			Bifidobacteria↓	

NA: Not available. ↑: Higher level; ↓: Lower level.

secondary to AP that might be attributable to gut translocation of intestinal bacteria [82,96]. Current guidelines recommend against routine antibiotic prophylaxis in AP [97]. Recent studies did not find benefits of antibiotic prophylaxis in reducing AP mortality, infections not involving the pancreas, or surgical interventions. The data on infections accompanying pancreatic necrosis in adults are conflicting[98,99].

The use of antibiotics, starting with carbapenems, quinolones, and metronidazole, has been advised in patients with AP and concomitant cholangitis symptoms, infected necrosis, or necrotizing pancreatitis accompanying a deteriorating clinical status[97]. Delaying surgical interventions decreases morbidity and mortality[100]. In some instances of AP, where infection is clinically suspected or confirmed, the use of antibiotics is recommended to avoid development of antimicrobial resistance. The predictive value of fine-needle aspiration for sampling and determination of bacterial sensitivities in diagnosing peri-pancreatic infection is comparable to that of clinical signs and imaging. The routine use of fine-needle aspiration is not recommended[101].

Early enteral nutrition is recommended in AP because it protects mucosal nutrition, the gut mucosal barrier, and gut-pancreas homeostasis[102]. In a previous randomized trial, decontamination of the gut with norfloxacin, colistin, amphotericin and standard AP therapy did not reduce mortality[103]. At present, selective gut decontamination cannot be recommended for AP patients.

Despite constant improvement in targeted therapeutics, a third of adult AP patients develop moderately severe AP and/or severe AP with SIRS, organ failure, and an increased risk of infection[104]. The gut mucosal barrier reduces the risk of infected pancreatic necrosis and thus helps to decrease mortality risk[85,105]. Microbiome-targeted therapies, such as genetic engineering of modified strains to outcompete pathogens, selective nutrient or prebiotic supplementation, or engineered bacteriophages, could steer the altered microbiome toward a healthy phenotype or change the course of critical illness[81]. Probiotics offer substantial health benefits and support the homeostasis of gut flora[81]. The most widely used probiotic bacteria in clinical trials are *Lactobacillus* and *Bifidobacterium*, which are easy to isolate from human feces or the

Table 6 Altered microbiome composition observed in acute pancreatitis of increasing severity

No.	Techniques used for microbiome profiling	MAP	MSAP	SAP	Ref.
1	qPCR (Fecal samples), performed only on MAP and SAP patients	*Enterococcus*↑ *Enterobacteriaceae*↑ *Bifidobacterium*↓	NA	*Enterococcus*↑ *Enterobacteriaceae*↑ *Bifidobacterium*↓	[79]
2	16S rRNA gene sequencing (Fecal samples)	*Finegoldia*↑	NA	*Acinetobacter*↑ *Stentrophomonas*↑ *Geobacillus*↑ *Bacteroides*↓ *Alloprevotella*↓ *Blautia*↓ *Gemella*↓	[83]
3	16S rRNA sequencing (Fecal sample)	*Enterobacteriaceae*↑ *Enterococcus*↑ *Bifidobacterium*↓	NA	*Enterobacteriaceae*↑ *Enterococcus*↑ *Bifidobacterium*↓ *Blautia*↓	[85]
4	16S rRNA gene sequencing (Rectal swab)	*Bacteroides*↑ *Escherichia/Shigella*↑ *Enterococcus*↑ *Finegoldia*↑ *Blautia*↓	*Bacteroides*↑ *Escherichia/Shigella*↑ *Enterococcus*↑ *Anaerococcus*↑ *Eubacterium hallii*↓	*Bacteroides*↑ *Escherichia/Shigella*↑ *Enterococcus*↑ *Eubacterium hallii*↓ *Acinetobacter*↓ *Stenotrophomonas*↓ *Bacteroides*↓ *Blautia*↓	[82]
5	Shotgun metagenomics (Fecal sample)	*Thermoprotei*↑ *Crenarchaeota*↑ *Streptococcus*↑ *Anaerostipes hadrus*↓	*Sulfolobus*↑ *Methanobrevibacter ruminantium*↑ *Methanosarcina - Thermophila*↑ *Anaerostipes hadrus*↓ *Escherichia coli*↑	*Sulfolobus*↑ *Methanomicrobiales - archaeon 53_19*↑ *Enterococcus*↑ *Blautia*↓	[86]

MAP: Mild acute pancreatitis; MSAP: Moderately severe acute pancreatitis; NA: Not available; SAP: Severe acute pancreatitis; ↑: Higher level; ↓: Lower level.

intestinal mucosa. Prebiotics are nondigestible foods required to propagate probiotics, and they stimulate the growth and activity of the healthy gut flora. Synbiotics are nutritional supplements that include both probiotics and prebiotics[106].

A randomized controlled trial by Pan *et al*[107] evaluated the ability of synbiotics to restore intestinal barrier damage and reduce the infection rate in early AP. A group of 45 patients were given either live or heat-inactivated *Lactobacillus plantarum* 299 with an oat fiber supplement as early enteral nutrition. Supplementation plus the symbiotic significantly reduced both pancreatic necrosis and surgical interventions[107]. A subsequent clinical trial included 62 severe AP patients treated with early enteral nutrition with four different prebiotics (inulin, beta-glucan, resistant starch, and pectin) together with four *Lactobacillus* probiotic preparations. The treatment resulted in a reduced incidence of SIRS and organ failure, supporting the use of early enteral symbiotic nutrition in severe AP[108]. Olah *et al*[109] randomized 45 AP patients to receive either a freeze-dried preparation containing 10^9 live *L. plantarum* 299 in each dose together with oat fiber or a heat-inactivated *Lactobacillus* controlled by nasojejunal tube for one week. Infected pancreatic necrosis and abscesses were significantly lower

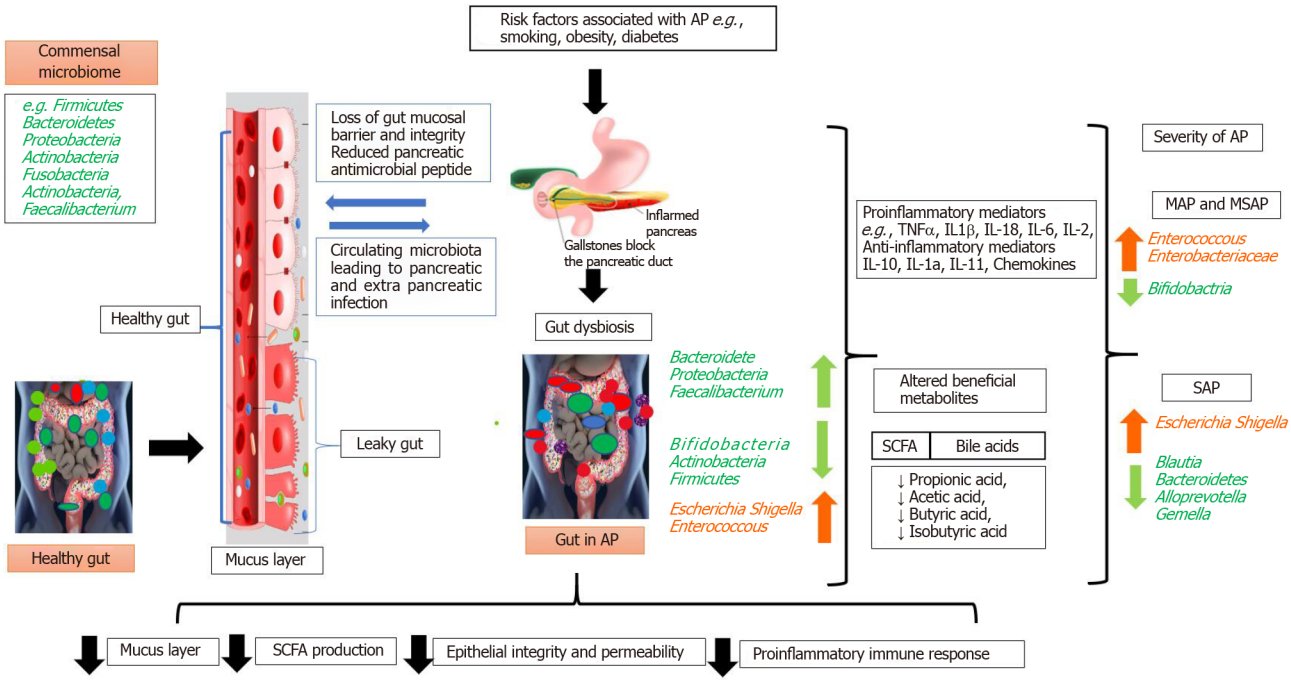


Figure 2 Role of the gut microbiota in inflammation of the pancreas and acute pancreatitis. Breakdown of the relationship between physiologic and pathogenic bacteria, the immune system, and the intestinal epithelial barrier leads to gut dysbiosis. Inflammation and gut dysbiosis causes the translocation of microbes to the pancreas. The translocation of bacteria results in pancreatic inflammation due to toxin diffusion and complications like fibrosis, digestive and absorption disorders, diabetes, and other metabolic disorders. AP: Acute pancreatitis; MAP: Mild acute pancreatitis; MSAP: Moderate severe acute pancreatitis; TNF: Tumor necrosis factor; IL: Interleukin; SAP: Severe acute pancreatitis; SCFA: Short-chain fatty acids.

in the treatment group than in the control group.

Other experimental pancreatitis studies in rat models confirmed the efficacy of *L. plantarum* spp. in reducing microbial translocation and as a possible alternative to antimicrobials[110]. Several studies have shown that probiotics containing *Faecalibacterium* and *Bifidobacterium* species had beneficial effects, including stabilizing the gut barrier, increasing anti-inflammatory responses, and attenuating bacterial translocation[111,112]. Some studies have not found any significant benefit or adverse effect of probiotics in severe AP. Still, it is essential to note the considerable patient and probiotic regimen heterogeneity in the published clinical trials of probiotics. The PROPATRIA Probiotics[113] in Pancreatitis Trial randomized 298 patients with predicted severe AP to either a multispecies probiotic mixture containing two different *Bifidobacterium*, three *Lactobacillus*, and one *Lactococcus* species or a placebo. The infectious complications in the two groups were similar, but the probiotic group had higher mortality (16% *vs* 6%) and incidence of bowel ischemia (6% *vs* 0%) compared with the placebo group. The high load of the probiotic mixture used in the study was thought to have been responsible for the increased mortality[114]. The findings highlight the challenges of supplementing the gut microbiome with beneficial microbial species in the setting of AP. Nevertheless, eight years later, the PROPATRIA trial was reevaluated by Bongaerts *et al*[115]. The team of researchers analyzed and addressed all shortcomings identified in the trial. PROPATRIA researchers contend that a lethal combination of predominantly proteolytic pancreatic enzymes and probiotic therapy was responsible for the high mortality rate, and that elevated levels of lactic acid produced by bacterial fermentation of carbohydrates significantly contributed to the high death rate. Additionally, one of them was the latency time in the first administration of probiotics; indeed, some patients were treated 24 h after onset of symptoms. Furthermore, there were errors in randomization; in fact, the onset of multi-organ failure was already present during admission in more patients in the first group than in the placebo group (41 patients *vs* 23 patients). Finally, last but not least, the team of researchers suggested that in future studies, when considering substituting probiotics in AP, it is necessary to assess the appropriate, effective doses of probiotics. However, caution should be mandatory to prevent bacterial overgrowth while conducting clinical trials in AP patients.

Previous studies have shown that *L. plantarum* decreased the occurrence of infective necrosis in AP patients[110] and that *Saccharomyces boulardi* spp. administered concomitantly with antibiotics such as ciprofloxacin decreased histopathologic scores in acute

necrotizing pancreatitis[111]. Animal studies have provided strong evidence in support of probiotic benefits in animal models of AP. A mixture of *Lactobacillus acidophilus, Streptococcus thermophilus,* and *Bifidobacterium lactis* given by oral gavage reduced pancreatitis, bacterial translocation to extra-intestinal sites, and mortality in male albino rats because of reduced duodenal bacterial overgrowth[112].

Injury of the GI barrier is a key event in the development of AP. Few studies have reported prevention of disruption of the intestinal barrier with modulation of gut microbiome balance. In one such study conducted in a mouse model of AP, *Clostridium butyricum*, a producer of small-chain fatty acids, which have immunomodulatory properties, reduced infiltration of neutrophils and dendritic cells in the pancreas and inhibited inflammatory responses mediated by NLRP3 and TLR4 signaling pathways in the pancreas and colon[27]. In summary, probiotics help to maintain gut homeostasis. Research should improve the designs of future studies, for example, by detecting a peculiar strain of microorganisms (*i.e.*, their type), standardizing the dose and duration of treatment, or standardizing the state of disease progression when considering to use in current therapy scenarios.

CONCLUSION

The gut microbiome plays a significant role in health and diseases. The resident microbiota in the human GI tract influences host metabolism, physiology, and immune system development. Disruption of this bacterial community results in GI disease. Ongoing medical and clinical research has produced a substantial body of evidence of a clear correlation between changes in the commensal microbiota and the occurrence of pancreatic disease. Application of biochemical, microbiological, and molecular biological methods have provided a description of the constituents of the gut microbiome in health and disease, their niches, and their physiological roles. Additional study is needed to explain whether microbial dysbiosis is a cause or an effect of diverse pathologies. The microbiome profile and changes in dysbiosis may influence an increase in AP severity during its clinical course.

Damage of the intestinal mucosal barrier allows migration of intestinal microbes to the blood or other tissues and organs, which enhances or aggravates AP. Changes in the resident species and abundance of the intestinal flora during AP are closely related to damage of the intestinal mucosal barrier system. Regulating the intestinal flora to repair the intestinal mucosal barrier and restore its function may be useful in AP treatment. Changes in the gut microbiota composition in AP include over-representation of opportunistic pathogens such as *Escherichia/Shigella* species and a significant decrease in the beneficial *Bifidobacterium* genus. Early dysbiosis of the gut microbiota, especially the depletion of small-chain fatty acid-producing bacteria, is probably associated with impairment of the gut barrier and increased AP severity.

The mechanisms of gut dysbiosis and the etiology of AP are not yet fully understood. The relationship of GI microbial symbiosis and AP are avenues for further research. The concomitant use of probiotics and antibiotics together with conventional treatment, such as surgery, radiotherapy, chemotherapy and targeted therapies, are further areas for research. In summary, the clinical significance of GI homeostasis during AP is emerging step by step. Thus, restoring the homeostasis of gut microbiota and stabilizing the gut barrier could be a promising therapeutic target in preventing AP progression. Challenges and specific problems that stand in the way to developing a research platform for understanding AP and its interaction with the microbiome need to be overcome. This review has explored the role of the gut microbiome in AP and the targeted use of probiotics, prebiotics, and synbiotics to maintain or restore GI microbial balance.

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MINIREVIEWS

Role of exercise in preventing and restoring gut dysbiosis in patients with inflammatory bowel diseases: A review

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Abstract

Inflammatory bowel diseases (IBD) include a spectrum of chronic inflammatory disorders of the gastrointestinal tract whose pathogenesis is yet to be elucidated. The intestinal microbiome has been studied as a causal component, with certain microbiotic alterations having been observed in subtypes of IBD. Physical exercise is a modulator of the intestinal microbiome, causing shifts in its composition that are partially corrective of those observed in IBD; furthermore, physical exercise may be beneficial in patients with certain IBD subtypes. This review studies the effects of physical exercise on the human gut microbiome while investigating pathophysiologic mechanisms that could explain physical activity's clinical effects on patients with IBD.

Key Words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Pouchitis; Microbiome; Exercise

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Core Tip: Inflammatory bowel diseases (IBD) are a spectrum of diseases that are characterized by their complex pathogenesis. The intestinal microbiome is thought to be a part of their pathogenesis, with certain alterations having been associated with

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IBD subtypes. Physical exercise is a modulator of the intestinal microbiome that has, furthermore, been associated with positive clinical outcomes in certain patients with IBD. Herein we discuss certain types of physical exercise, their effect on the intestinal microbiome, and its clinical effects on patients with IBD, as well as investigating underlying pathophysiologic mechanisms that could mediate the observed associations.

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INTRODUCTION

Inflammatory bowel disease (IBD) comprises a spectrum of chronic inflammatory diseases that primarily but not exclusively affect the gastrointestinal tract, including ulcerative colitis (UC), Crohn's disease (CD), and other related conditions[1]. Their pathogenesis is classically thought to include interactions between genetic, immune-mediated, and environmental factors[2]. Studies on the molecular epidemiology of IBD have shown that the gut microbiome composition is a biomarker of prognostic importance for CD and UC[3]. Other molecular phenotypes include *NOD2*, *MHC*, and *MST1* genotypes, which are correlated to disease location and activity, microRNA miR-215 levels, and DNA methylation, which are correlated to disease activity, *FOXP3* haplotype, which is of prognostic importance, and oncostatin M and IL-1β levels, which predict response to anti-tumor necrosis factor therapy[3,4]; furthermore, serum C-reactive protein levels correlate to disease activity but are generally nonspecific[4]. Increased serum miR-595 and miR-1246 levels are associated with active IBD[4]. Serum interleukin (IL)-2 and IL-6l and positivity for anti-bacterial flagellin, anti-outer membrane porin C, anti-A4-Fla2, and anti-Fla-X antibodies predict recurrence of disease[4]. The heterogeneity of IBD phenotypes underlines the need for new markers that can subclassify, diagnose, inform prognosis, and guide IBD treatment; but owing to the low sensitivity or specificity of known molecular markers, current knowledge is far from adequate to support their use in everyday clinical practice, a limitation that includes the microbiome as biomarker[3,4].

Early studies on animal models have shown that immune cells could not cause inflammation in the absence of intestinal bacteria, therefore suggesting a putative role for the intestinal microbiome in the induction and/or maintenance of local inflammation and disease[5]. This was further supported by the observation that intestinal inflammation in IBD was greatest in parts of the bowel richer in bacteria[2]. Further studies have demonstrated that certain patterns of microbiotic alterations, including increases or reductions in the plethora of bacterial, fungal, and viral species, were likely linked to the risk for IBD[2].

Physical exercise is a possible modulator of intestinal microbiome composition, altering the functional activity of the gut ecosystem. Exercise is associated with increased biodiversity and a beneficial metabolic function, while exhaustive exercise training might be associated with dysbiosis of the gut microbiota, promoting negative metabolic effects and inflammation[6,7]. Thus, physical activity/exercise has been studied as a significant modifier of the intestinal microbiome in animal[8,9] and human[6,7,10] studies. Specifically, more active individuals' microbiomes tend to harbor a higher abundance in *Akkermansia muciniphila*, a health-promoting species, as well as decreased Bacteroidetes bacteria and increased bacterial diversity[11,12]. Specific types of physical exercise have not only been associated with microbiotic signatures, but also with a reduction in endotoxemia and serum inflammatory markers [11,13,14]. These alterations persist as statistically significant, even when normalizing for age, weight, body composition, and nutritional habits as confounding factors[15]. Nevertheless, it should be noted that researchers have also reported a lack of correlation between a certain type of exercise and changes in the microbiome[16,17]; moreover, some contradictions as to the observed patterns of microbiotic alterations associated with exercise are evident[18]. This ambiguity is, however, expected to be partly clarified when common definitions and detailed description of exercise charac-

teristics [*i.e.* frequency, intensity, type (mode), time (duration), and volume/dose (duration x intensity)] and methods of fecal sampling are utilized among the same study groups.

Given the established pathobiological role for the microbiome in IBD and taking into account the more recent data assessing patterns of microbiotic alterations associated with exercise, this review aims to summarize and clarify the important findings linking dysbiosis in IBD to physical activity, with a focus on preventative medicine and therapeutics.

METHODLOGY

Our literature search utilized the PubMed literature database. Search keywords included "inflammatory bowel disease", "IBD", "ulcerative colitis", "Crohn's disease", "indeterminate colitis", "microbiome", "microbiota", "physical activity", "exercise", as well as combinations of the aforementioned with the AND/OR operators. Articles were first filtered by title, followed by abstract screening, and the remaining were finally selected based on their full text.

EXERCISE AND ITS INFLUENCE ON THE MICROBIOME

In healthy mice, *Allobaculum* and *Clostridiales* are more abundant in exercised mice than in controls[19]. In diabetic mice, total intestinal bacteria and Enterobacteriaceae are lower in the exercise groups than in diabetic controls[9]. In high-fat diet obese mice, exercise increases Bacteroidetes as well as increases the Bacteroidetes/Firmicutes ratio in the cecum and colon[20]. In an early obesity and non-alcoholic fatty liver disease model, where male rats fed a control or a high-fat diet, a combined aerobic and resistance exercise training resulted in increased *Parabacteroides*, *Bacteroides*, and *Flavobacterium* genera, while *Blautia*, *Dysgonomonas*, and *Porphyromonas* exhibited an opposite pattern[8] (Table 1).

Several factors that modify the human gut microbiome have been identified; those include country of residence, specific genotypes (such as those affecting the ABO antigens), delivery by caesarian section, diet, cigarette smoking, breastfeeding, gastroenteritis, increased hygiene, use of antibiotics, obesity, immune response, as well as physical exercise[2,13,21,22]. Human studies have shown that sedentary individuals have a predominance of *Bacteroides* and *Parabacteroides* in their gut microbiome, while participants with a higher level of activity, as gauged by accelerometers, have a predominance of *Coprococcus*, *Blautia*, and *Eubacterium*[23]. Women with an active lifestyle have a higher proportion of *Faecalibacterium prausnitzii*, *Roseburia hominis,* and *Akkermansia muciniphila* bacteria in their gut than sedentary women[24]. Aerobic brisk walking increases *Bacteroides* species in healthy elderly women[25]. Endurance training has been observed to reduce *Streptococcus*, *Proteobacteria*, *Porphyromonadaceae*, *Odoribacter*, *Desulfovibrionaceae*, and *Enterobacteriaceae* and to increase *Verrucomicrobiaceae*, *Bifidobacteriaceae*, *Dorea*, *Anaerofilum*, and *Akkermansia* bacteria in overweight women[26]. In obese children, a strength and endurance combined training program led to an increase in *Blautia*, *Dialister,* and *Roseburia* species, accompanied by a reduction in inflammasome activation[14]. In elderly men, endurance exercise has been observed to reduce *Clostridium difficile* and increase intestinal populations[27] Male elite rugby players have been found to have a more diverse microbiome than body mass index (BMI)-matched controls, with the athletes having higher proportions of *Akkermansiaceae* and *Akkermansia* than high-BMI controls and lower proportions of *Lactobacillaceae*, *Bacteroides,* and *Lactobacillus* than low-BMI controls[10]. In swimmers, a reduction in training volume is accompanied by a significant reduction in *Coprococcus* and *Faecalibacterium* populations[28]. In marathon runners, *Lentisphaerae* and *Acidobacteria* increase in intestinal population after running[29]. Ultra-endurance exercise has been observed to increase butyrate-producing bacteria, such as *Subdoligranulum* and *Roseburia hominis*, which are thought to reduce intestinal inflammation by producing butyrate[30]. In martial arts athletes, *Parabacteroides, Phascolarctobacterium, Bilophila, and Oscillibacter* are higher in higher-level athletes than in lower-level ones, with *Allisonella*, *Citrobacter,* and *Megasphaera* found at lower levels[31]. Other researchers have reported no change in gut bacterial diversity or composition after short-term high-intensity interval training in lean and overweight men[16]. Exercise has also been found to induce microbial transformations in the context of damaging intestinal conditions, such as a high-fat diet and toxic substances[32] (Table 1). These changes

Table 1 Effect of exercise on the gut microbiome

Ref.	Study subject	Sample type	Exercise protocol	Exercise *vs* controls	Notes
Lambert *et al* [9]	Mouse, type 2 diabetic, (C57BL/KsJ-leprdb/leprdb)	Cecal matter	Treadmill, 5 d/wk, 66 min/d, 2.87 m/min	↑ Clostridium leptum ↑ Lactobacillus ↓ Total bacteria ↓ Bacteroides ↓ Bifidobacterium ↓ Methanobrevibacter ↓ Prevotella	↑ Bifidobacterium with exercise in non-diabetic mice
Campbell *et al*[19]	Mouse C57BL/6NTac, male	Fecal matter from the distal colon	Free running wheel	↑ Allobaculum ↑ Clostridiales ↑ Faecalibacterium prausnitzi	Normal-diet, not observed in a high-fat diet, except for Faecalibacterium prausnitzi
Denou *et al* [20]	Mouse, C57 BL/6, high-fat diet, obese	Feces from anal area, then full intestinal sampling	Treadmill, 6 wk total, 3 d/wk, 1 h/d, 17 m/min at 5% grade for 2 min + 2 min rest, increase by 1 m/min every week	↑ Bacteroidetes/Firmicutes ratio in the cecum ↑ Bacteroidetes/Firmicutes ratio in the rectum	-
Carbajo-Pescador *et al*[8]	Juvenile male Wistar rats on early obesity and non-alcoholic fatty liver disease onset	Fecal matter	Treadmill, 11 wk total, 60 min/d combined aerobic and resistance training (10-min running; eight 2-min progressive incline run from 10°-25° at 20-25 cm/s /1 min rest; 30 min aerobic exercise)	↑ Parabacteroides ↑ Bacteroides ↑ Flavobacterium genera ↓ Blautia ↓ Dysgonomonas ↓ Porphyromonas	
Clarke *et al* [10]	Human, rugby player, male	Fecal matter, self-collected	Rugby training, capacity determined by EPIC-Norfolk questionnaire	↑ Akkermansia (than high-BMI controls) ↓ Bacteroides (than low-BMI controls) ↓ Lactobacillus (than low-BMI controls)	-
Bressa *et al* [24]	Human, female, premenopausal, BMI 20-25 kg/m^2	Fecal matter, self-collected	No forced exercise, physical activity level gauged by accelerometers	↑ Akkermansia muciniphila ↑ Faecalibacterium prausnitzii ↑ Roseburia hominis	-
Munukka *et al*[26]	Human, female, sedentary, BMI > 27.5 kg/m^2	Fecal matter, self-collected	Ergometer, Weeks 1-2: at 60 rpm, low intensity, 3 d/wk, 40 min/d Weeks 3-4: 3 d/wk, 50 min/d, every other session 3 10-min intervals of moderate-intensity cycling, the rest low intensity Weeks 5-6: 3 d/wk, 60 min/d, four 10-min moderate intensity intervals, the rest low intensity	↑ Akkermansia ↑ Anaerofilum ↑ Bifidobacteriaceae ↑ Dorea ↑ Verrucomicrobiaceae ↓ Desulfovibrionaceae ↓ Enterobacteriaceae ↓ Odoribacter ↓ Porphyromonadaceae ↓ Proteobacteria ↓ Streptococcus	-
Taniguchi *et*	Human, male, age > 60	Fecal matter,	Cycling, Weeks 1-2: 3 d/wk, 30	↓ Clostridium difficile	-

al[27]	yr, healthy	self-collected	min/d, 60% of VO_2peak (week 1), 70% of VO_2peak (week 2) Weeks 3-5: 3 d/wk, 45 min/d, 70% of VO_2max (week 3), 75% of VO_2max (weeks 4-5)	↑ Oscillospora	
Zhao *et al*[29]	Human, marathon runners	Fecal matter, self-collected	The 2016 Chongqing half marathon, before and after the race	↑ Acidobacteria ↑ Lentisphaerae	Post- *vs* pre-running
Castellanos *et al*[23]	Human	Fecal matter, self-collected	No forced exercise, physical activity level gauged by accelerometers	↑ Blautia ↑ Coprococcus ↑ Eubacterium ↓ Bacteroides ↓ Parabacteroides	-
Keohane *et al* [30]	Human, male, athlete	Fecal matter, self-collected	Rowing race, 33 d 22 h, 151.8 km per day	↑ Roseburia hominis ↑ Subdoligranulum	Post-ultra-endurance exercise
Liang *et al* [31]	Human, martial arts athlete	Fecal matter, self-collected	Martial arts, athletes, divided into higher- and lower-level based on General Administration of Sport of China criteria	↑ Bilophila ↑ Oscillibacter ↑ Parabacteroides ↑ Phascolarctobacterium ↓ Allisonella ↓ Citrobacter ↓ Megasphaera	Higher- *vs* lower-level athletes
Morita *et al* [25]	Human, female, age > 65 yr, sedentary	Fecal matter, self-collected	Trunk strengthening training, 12 wk, 1 h/wk: 5-10 min of warm-up + 45 min of targeted resistance training of trunk muscles + 5 – 10 cool-down and at-home exercise daily	↑ Bacteroides	After 12 wk of aerobic training
Hampton-Marcell *et al* [28]	Human, age 18-24 yr, swimmers	Cotton swab sample	Self-reporting of daily swimming distance and duration during daily practice	↑ Coprococcus ↑ Faecalibacterium	Before *vs* after reduction of training volume
Quiroga *et al* [14]	Human, age 7-12 yr, obese	Fecal matter, self-collected	Strength and endurance training 12 wk, 2 d/wk: Warm-up on an ergometer for 7 min, low-medium load, 60 rpm Third minute onwards, a sprint of 30s at 3’30”, 4’30”, 5’30”, and 6’30” Strength exercises for five muscle groups, initially 3 sets of 12 repetitions at 30% 1RM, up to 3 sets of 8 repetitions at 70% 1RM Cool-down at an elliptical cardiovascular device, 7 min, 50 rpm, 4 min low-medium load + 3 min high load	↑ Blautia ↑ Dialister ↑ Roseburia	After a 12-wk strength and endurance training program
Rettedal *et al* [16]	Human, male, age 20-45 yr	Fecal matter, self-collected	9 sessions of high-intensity interval training on non-consecutive days over 3 wk: 60 s cycling at VO2peak 75 s rest 8 intervals initially, up to 12 intervals by the end of the protocol	No significant changes in composition	Before and after high-intensity interval training

BMI: Body mass index.

could in part be attributed to increased gut motility during exercise, which promotes shedding of loosely bound bacteria and the growth of health-promoting species[33].

ROLE OF THE MICROBIOME IN IBD

Patients with CD have a diminished diversity of the fecal intestinal microbiome, with Lachnospiraceae, Bacteroidetes, and the species *Clostridium leptum* being decreased, with Proteobacteria, Actinobacteria, and the genus *Prevotella* being increased[13]. Patients with inflammatory bowel disease also have increased *Fusobacterium*, *Pasturellaceae*, *Ruminococcus gnavus*, *Veillonellaceae*, *Candida albicans*, *Candida tropicalis*, *Clavispora lusitaniae*, *Cyberlindnera jadinii*, *Kluyveromyces marxianus,* and *Caudivirales* in their gut microbiota, as well as decreased *Bacteroides*, *Bifidobacterium*, *Clostridium* XIVa, *Clostridium* IV, *Faecalibacterium prausnitzii*, *Roseburia*, *Suturella*, and *Saccharomyces cerevisiae* [2]. Therefore, it can be argued that intestinal dysbiosis may be a component of IBD pathogenesis[34].

The involvement of the microbiome in the pathogenesis of IBD is further supported by the effectiveness of antibiotic therapy in the treatment of certain IBD phenotypes, such as perianal CD and pouchitis, and in the prevention of postoperative relapse in patients with CD[2].

Fecal microbiota transplantation involves the transfer of feces from a donor to the GI tract of a recipient, as an attempt to enrich the recipient's gut microbiota and correct any dysbiosis. Fecal microbiota transplantation is currently systematically used in the treatment of *Clostridium difficile* colitis[35]. Some researchers have also investigated its implementation in IBD therapeutics, with some promising findings having been reported, although no concrete conclusions can yet be drawn about its efficacy and safety, plausibly owing to the dissimilarities between the associated studies[35].

ANIMAL CLINICAL DATA

Gut microbiota transplant from exercise-trained mice leads to a reduction in inflammatory markers in the distal colons of sedentary mice, combined with an attenuated colitis histology score[36]. In mouse models of colitis, voluntary treadmill exercise has been found to reduce inflammation while forced exercise exacerbates tissue damage and leads to increased mortality[37]. The same researchers then attributed part of this effect to an increase in *Tenericutes* bacteria in the large intestine in the forced-exercise group, since the family *Mollicutes*, a member of the phylum *Tenericutes*, has been linked to UC in humans[38]. In another study, exercise was shown to ameliorate the symptoms of chemically induced colitis and to alter significantly gut microbiota, with decreased populations of *Bacteroides vulgatus* and increased numbers of *Akkermansia muciniphila*[39]. In mice born without a normal mucus layer in their intestines due to a genetic knockout of mucin-2, exercise neither significantly alters the gut microbiome nor reduces the severity of chronic colitis, in contrast to wild-type mice where both effects have been observed[40].

HUMAN CLINICAL DATA

Based on current literature, it is not clear whether dysbiosis is a cause or a result of IBD[13]; however, some conclusions could be drawn from certain scientific findings: Disease activity is mostly focused on bowel segments where the fecal stream is slower and bacterial populations are higher[2]. Mutations in genes affecting the functions of intestinal Paneth cells (*e.g.*, *NOD2*), which defend the small intestine against bacteria, are risk factors for IBD[2]; moreover, exposure to antibiotics in early life is linked to IBD later in life[13]. Antibiotics are, however, effective in certain conditions involving IBD, such as in inflammation of the ileoanal pouch after colectomy indicated by IBD-related complications[41]. Surgical fecal diversion is beneficial in the treatment of CD, as bowel segments excluded from the fecal stream tend to show remission[2]; furthermore, probiotics and fecal transfer are a therapeutic option for inducing and maintaining IBD remission[13]. These findings suggest that there exists a causal component to the microbiotic patterns associated with IBD, despite the inconsistencies between observed microbiotic changes as reported by different studies[13].

Regarding the probable protective effect of exercise in IBD, it is thought to stem from anti-inflammatory actions[6]. These include the secretion of myokines by skeletal muscles, such as myostatin, irisin, IL-15, brain-derived neurotrophic factor, myonectin, decorin, and secreted protein acidic and rich in cysteine, mediators with autocrine, paracrine, and endocrine anti-inflammatory actions[42]; moreover, in obese humans, exercise has been found to alter the gut microbiome and reduce endotoxemia, as

measured by the levels of the endogenous protein lipopolysaccharide binding protein [11], thus suggesting another probable anti-inflammatory effect for physical activity. Exercise training has also been found to reduce the levels of NLR Family Pyrin Domain Containing 3 and caspase 1, proteins that participate in the inflammasome activation pathway, in obese children[14].

Several mechanisms have been proposed by which exercise may influence gut microbiota. These include crosstalk between muscles and the gut microbiota through the 5′ adenosine monophosphate-activated protein kinase and fasting-induced adipose factor pathways as well a reduction of fecal bile acids, an increase in production of short-chain fatty acids, an increase in gut luminal immunoglobulin A, a reduction in luminal transit time, and the activation of the stress hypothalamic-pituitary-adrenal axis, effects found to be produced by exercise[43-46].

The functions of a healthy microbiome include metabolic functions, such as the production of anti-inflammatory short-chain fatty acids, vitamin K, and biotin, protective effects, such as induction of secretions that attack pathogenic bacteria, triggering of mucosal proliferation through the Toll-like receptor pathway, inhibition of adhesion of pathogenic bacteria, and trafficking of neutrophils, as well as trophic functions, *i.e.* protecting the intestinal mucosa from immune-mediated damage[46-48]. Part of the pathogenesis of IBD is thought to include a loss of these effects, probably caused by a damaging shift to the microbial composition in the gut[1]. These include reductions in Bacteroidetes, *Clostridium leptum*, *Prevotella*, *Bifidobacterium*, and *Roseburia* as well as increases in Proteobacteria, as mentioned above. All of these alterations are restored by exercise interventions in animal and human studies (Table 1), suggesting a plausible mechanism for the beneficial effect of exercise in IBD.

Current exercise guidelines from the American Heart Association for adults aged 18 years to 65 years of age recommend moderate-intensity aerobic exercise for at least 30 min, 5 d a week or vigorous-intensity exercise physical activity for 20 min, 3 d a week [49]. No specific guidelines exist for patients with IBD, however, evidence suggests that mild-to-moderate exercise harbors multiple benefits for patients with at least mild IBD, and excessive exercise could pose hazards for patients' health[41]; therefore, physicians should be cautious when prescribing exercise for patients with IBD, being on the lookout for exercise addiction[41].

Clinical data studying the association of IBD with exercise suggest that sedentary occupations confer a higher risk for IBD than more physically demanding occupations, as found in a retrospective study of German employees[50]; moreover, both CD and UC have been associated with low physical activity during childhood[51]. Exercise has been reported to decrease the risk of relapse in patients with IBD in remission[52]. Mild-to-moderate exercise is beneficial in patients with at least mild IBD[41]. A recent study reported that prolonged moderate-intensity walking did not appear to increase blood cytokine levels in patients with IBD more than it did in healthy controls, and fecal calprotectin levels were found to be comparable between patients who walked and patients who did not walk, suggesting that exercise does not cause exacerbation of IBD[53].

CONCLUSION

The findings of the present review imply that there exists a promising field of research regarding exercise-induced changes of the microbiome in IBD. What needs to be elucidated is whether the microbiome is a passive "bystander" in the systemic effects induced by physical activity, *i.e.* observing and reacting to activity-related systemic metabolic and endocrine signals by altering its composition, or whether it is a necessary physiological intermediate in the restoration of immune tolerance and normal gastrointestinal function in the context of IBD. Further research should also focus on disease determinants, such as age, sex, type, localization, histology, refractory phenotype, disease activity, molecular markers, and performance status, which could affect the disease's response to certain types of physical exercise. Besides, considering that there is not only one optimal microbiota composition for the IBD patients, more studies are also needed to reveal how microorganisms interact with each other and with their host to identify different healthy microbiota schemes and an optimal, potentially personalized, dose of exercise for these patients. Lastly, an interesting field might exist for the microbiome as an index predictive or indicative of exercise-induced amelioration of IBD clinical symptoms, as part of current research on the molecular epidemiology of IBD.

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MINIREVIEWS

Gastrointestinal mucosal immunity and COVID-19

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Abstract

As the gastrointestinal tract may also be a crucial entry or interaction site of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the role of the gut mucosal immune system as a first-line physical and immunological defense is critical. Furthermore, gastrointestinal involvement and symptoms in coronavirus disease 2019 (COVID-19) patients have been linked to worse clinical outcomes. This review discusses recent data on the interactions between the virus and the immune cells and molecules in the mucosa during the infection. By carrying out appropriate investigations, the mucosal immune system role in SARS-CoV-2 infection in therapy and prevention can be established. In line with this, COVID-19 vaccines that stimulate mucosal immunity against the virus may have more advantages than the others.

Key Words: Mucosa; Gut mucosa; Mucosa-associated lymphoid tissue; SARS-CoV-2; COVID-19; Secretory immunoglobulin A; Gut microbiota

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respiratory syndrome coronavirus 2. Given the complex interactions between the virus and the mucosal immune system after exposure, additional research is needed to elucidate the immune mechanisms and processes in the gut mucosa. The hallmark of all immune responses is the recruitment of various immune cells, such as neutrophils, dendritic cells, macrophages, and T cells in the gut mucosa. However, the mucosal inflammatory response could change intercellular space between enterocytes, leading to an increase in intestinal permeability that allows various bacterial antigens and toxins to enter the bloodstream, further complicating the disease state of coronavirus disease 2019 patients.

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INTRODUCTION

It is well-established that nasopharynx-associated lymphoid tissue (NALT) and mucosa-associated lymphoid tissue (MALT) are first-line defenses. Therefore, airborne infections start by penetrating the upper airway mucosa, where a higher viral load is found, compared with the throat. NALT is involved in the induction of the immune response towards the microorganisms by promoting the differentiation and activation of immune cells such as Th1- and Th2 cells, dendritic cells, macrophages, resident microfold M cells, innate lymphoid cells, immunoglobulin (Ig)A-switched B cells, as well as immune mediators and molecules (*i.e.* beta-defensins, galectins, collectins, and cytokines)[1]. The same goes for the gut mucosal immune system.

It is thus not surprising that NALT exerts "gate control" on many infections that penetrate the mucosa, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[2]. SARS-CoV-2 acts cytopathically at the mucosal level by inducing injury and death of the infected cells. That can be accomplished by pyroptosis as a consequence of activation of host-cell released damage-associated (DAMPs) and viral pathogen-associated molecular patterns (PAMPs) and innate immunity along with the secretion of many cytokines [interleukin (IL)-6, interferon (IFN)-gamma, MCP1, and IP-10)][1]. On the other hand, pattern-recognition receptors and their soluble forms are mainly involved in SARS-CoV-2 infection[3,4].

Amongst the immune molecules, the collectins have a significant role in recognizing glycoside structures of the virus. For example, deficiency of mannose-binding lectin (MBL) has been associated with increased susceptibility to viral infections, including SARS-CoV[5]. Additionally, MBL can inhibit S protein by binding to it and inhibiting structural S rearrangements needed for optimal infection, thus leading to reduced virulence of SARS-CoV-2. Furthermore, as the expression of MBL declines with aging, older adults may be more prone to infection because of lack of an effective innate response[5].

Additionally, the presence of natural, pre-existing IgM and IgA antibodies produced in the absence of any antigen, provides the first-line defense[6]. Furthermore, some of the natural antibody subsets can recognize ABO blood-group antigens, which are expressed on many epithelial cells, including the lung[7]. As enveloped viruses like SARS-CoV-2 are highly glycosylated, it is thought that when virions reproduce in the alveolar epithelial cells in people with group A or B blood, those antigens may be expressed on their envelope. Thus, one may suggest that natural antibodies against A and B antigens may be protective[8]. Studies have so far revealed that anti-A antibodies can inhibit S protein binding to angiotensin-converting enzyme 2 (ACE2) receptors when the host cells express A antigen. Individuals with group O blood have a reduced risk of infection compared with those who have non-O blood groups, and those with group A blood are prone to severe coronavirus disease 2019 (COVID-19)[8].

EPIDEMIOLOGICAL SIGNIFICANCE OF MUCOSAL PENETRATION AND REPLICATION FOR THE SPREAD OF SARS-COV-2

COVID-19 is an infectious disease in which the primary mode of transmission of its causative agent, SARS-CoV-2, is by transfer of saliva microdroplets between people in close contact. The microdroplets are produced while coughing, sneezing, or talking. Infection by contact with contaminated surfaces followed by touching the face is less common. Most microdroplets fall to the ground or surfaces and are not effective over long distances. The first 3 d after the onset of symptoms is when the patient is most contagious. Transmission may occur before symptoms appear. Asymptomatic people may thus be contagious, too[9].

COVID-19 is an airborne viral disease. It was shown that SARS-CoV-2 penetration into the upper airways is the first step of the infection, as higher viral loads were found in nasal swabs than throat swabs[10,11]. The same distribution as in symptomatic patients was observed in asymptomatic patients, implicating the nasal epithelium as a portal for initial infection and transmission[12]. The nose is a critical component of mucosal immunity, providing protection in the upper airway. It is involved both in host protection and immune homeostasis between the commensal microbiota and invading pathogens. The mucosal immune system is the first line of physical and immunological defense against invading pathogens[13]. Current evidence indicates that SARS-CoV-2 enters the human body mainly through the ACE2 + transmembrane protease/serine subfamily member 2 (TMPRSS2) + nasal epithelial cells. The initial host response to this pathogen begins in the NALT system[1].

VIRAL FACTORS OF SARS-COV-2 AND IMPACT ON THE MUCOSA

Coronaviruses are enveloped, positive-sense single-stranded RNA viruses that are members of families *Coronaviridae*, order Nidovirales. There are four known genera, *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*. SARS-CoV-2 belongs to the family *Coronaviridae* and genus *Betacoronavirus*[14,15]. Diseases caused by coronaviruses comprise symptoms that range from mild respiratory illness like the common cold to severe infections causing death. These viruses can infect humans, mammals, and avian species, including farm and companion animals (pets). Hence they represent not only a challenge for public health but also are of veterinary and economic concern. From the beginning of the 21st century, the SARS epidemic in 2002-2003, the Middle East respiratory syndrome (MERS) in 2012, and the emergence of the new SARS-CoV-2, are examples of human infections caused by coronaviruses[16,17].

Coronaviruses are spherical, enveloped RNA viruses containing an impressively large (25 kb to 32 kb), nonsegmented, single-stranded, positive-sense RNA genome, which is the same sense as the messenger RNA (mRNA) found in cells. The genome codes four main structural proteins, nonstructural proteins (NSPs), and accessory proteins. The structural proteins, which include the spike (S), nucleocapsid (N), membrane (M), and envelope (E) proteins, play essential roles in the arrangement of the virus particles and other aspects of the viral life cycle[18]. Among the structural proteins, the most important is S protein, which is required for viral entry as it binds to the target cell receptors and initiates fusion with the cell membrane[19].

SARS-CoV-2 spike (S) protein is a large glycosylated transmembrane, homotrimeric protein. Each monomer has a molecular mass of about 150-200 kDa[18]. Each subunit of the protein consists of two functionally distinct domains, S1 and S2. S1 forms the bulb portion of the spike protein on the virion surface. S2 attaches the S proteins to the viral membrane. The receptor-binding domain is located on S1; S2 is necessary for membrane fusion to allow the viral cell entry[20]. Transmembrane ACE2 is the functional host receptor for SARS-CoV-2[21]. ACE2 is widely expressed in the ciliated, goblet, and surfactant-producing type-2 alveolar cells of the lungs, intestinal, cardiac, and vascular endothelia, the kidney, and the liver[22].

Other receptors, such as DC-SIGN, L-SIGN, Neuropilin-1, furin, and cathepsin B and L can serve as portals of virus entry into the cell. Taken together, the findings provide a possible explanation for the occurrence of COVID-19 complications in organs expressing those receptors. The binding of S protein to the host ACE2 receptor alone is not enough for the viral fusion. The spike protein needs to be cleaved by cell surface serine proteases at specific sites (S1/S2 boundary and S2`), releasing the S1 domain, which subsequently activates the S2 domain, leading to fusion of the viral cellular membranes[23]. Host-cell surface serine proteases shown to cleave the S proteins include, but are not limited to, TMPRSS2, furin, and trypsin. Both ACE2 and

TMPRSS2 (also furin) are highly expressed in the gastrointestinal (GI) tract, particularly intestinal epithelial cells[24]. The primary entry site of SARS-CoV-2 is host lung cells. Nevertheless, the GI may also be a crucial entry or interaction site. SARS-CoV-2 viral particles are preferentially released apically and not at the basement of the airway cells. Thus, the released SARS virus may be removed by mucociliary clearance with access to the GI *via* luminal exposure. Moreover, the early appearance of gastrointestinal symptoms such as nausea, vomiting, abdominal pain, and diarrhea in almost 30% of COVID-19 patients supports this hypothesis.

Viral factors of SARS-CoV-2 and host innate immune response

After the fusion with the cell membrane, the viral genome is released into the host-cell cytoplasm, and the highly controlled process of viral RNA replication and transcription occurs[23]. The virus interacts with cellular compartments and proteins to make its RNAs and proteins. It has been shown that some viral proteins influence critical host-cell processes such as apoptosis, necrosis, innate immunity, and others. One of the structural proteins, nucleocapsid (N) protein, despite its central role in binding to the viral RNA genome, was shown to inhibit type I IFN production and signaling. Recent studies provide evidence that NSP1 (participates in host-cell mRNA degradation and translation inhibition[25]. Hence, the translation of vital cellular proteins, including type I IFN, is shut down, allowing viral RNA to be translated effectively[26]. That may be the reason why NSP1 is cleaved and activated immediately after the production of polypeptide pp1a.

PLpro and Mpro (3CLpro) viral proteases are necessary for the proteolytic cleavage of the polyproteins (pp1a and pp1ab)[20]. Furthermore, it has been shown that the proteases play a role in inhibition of type I IFN signaling. PLpro is responsible for only a few cleavage events in pp1a, but it also can act as a deubiquitinase and deISGylating (removal of IFN-stimulated gene 15 from proteins), which are enzyme activities that lead to evasion and the initial steps of the antiviral response[27,28].

During viral RNA replication and transcription, various PAMPs are produced in the form of double-stranded RNA intermediates. It has been suggested that some of the viral proteins (*i.e.* E, N, NSPs) are involved in the formation of convoluted membranes and double-membrane vesicles to create a protective microenvironment for genomic RNA replication and transcription of subgenomic mRNAs[23]. Furthermore, the PAMPs are recognized by endosomal Toll-like receptors (TLR 3, 7, 8) or cytoplasmic RNA PRRs, such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5). The proper function of TLRs, RIG-I, and MDA-5 is crucial for host-cell survival, as the receptors provide a first-line defense against infections[29-31]. The viral genome attachment to the receptors, especially the TLRs, gives rise to innate immune response signaling pathways. In leukocytes (*e.g.*, dendritic cells, macrophages, natural killer cells, T cells of the adaptive immune system, and B cells), interaction with viral RNA triggers innate immune responses and development of acquired antigen-specific immunity[31]. The innate immune system senses the foreign viral materials that are possibly pathogenic, which initiates downstream signaling to the nucleus, and in turn promotes the expression of types I and III IFNs and other proinflammatory cytokines. Once activated, the IFNs induce a cascade of cellular and molecular events that lead to the suppression of viral replication and reduction in the number of infected cells. Many viral proteins influence the IFN signaling pathway, thus providing a favorable environment for virus development. PLpro, NSP1, ORF3b (a viral accessory protein), and N inhibit two proteins (IRF3 and IRF7) required for INF transcription, thus inhibiting the first steps in the innate immune response against the virus[29-31]. ORF3a and ORF3b are viral proteins that induce caspase-independent necrotic cell death and initiate an inflammatory cascade through activation of the NLRP3 inflammasome[32].

It should be noted that necroptosis and pyroptosis are highly inflammatory mechanisms of cell death that lead to increased secretion of proinflammatory cytokines and chemokines, thus contributing to further tissue damage. Several studies have found that expression of N, E, M, ORF3a, ORF3b, ORF7a, ORF8a, or ORF9b proteins in various cell lines triggered apoptosis through cytochrome C release and caspase-dependent pathways. Apoptosis is a form of noninflammatory cell death that often serves as a host response during viral infection. At the moment, it is not yet clearly elucidated what the exact role of SARS-CoV-2 induced cell death is. It may be an exit strategy to increase viral spread, a form of immune evasion, or just an indirect effect of viral replication on the host cell cycle[23].

Proteome analysis of SARS-CoV-2 has shown that viral proteins interact with more than 300 host-cell proteins, leading to cellular mRNA degradation, inhibition of translation, inhibition of IFN production and signaling, induction of apoptosis and

necrosis, and other activities. The hallmark of all these functions is an evasion of host innate immune responses that could facilitate viral spreading to nearby cells. The changes in infected enterocytes could result in the recruitment of neutrophils, dendritic cells, macrophages, and T cells in the gut mucosa. The mucosal inflammatory response could change the intercellular space between enterocytes, leading to an increase in intestinal permeability that provides an opportunity for bacterial antigens and toxins to enter the bloodstream and further complicated the disease state of COVID-19 patients.

SARS-COV-2 AND MUCOSAL IMMUNITY OF THE GI

An effective and powerful adaptive immune response follows the early antiviral innate response in the mucosa. Expansion of CD4+ T-helper cells, CD8+ cytotoxic T cells, and plasma cells simultaneously with the ongoing innate immune response is critical for virus elimination[33]. However, many factors can alter the immune response and control of the viral replication cycle. External factors such as smoking, pollutants, temperature, humidity, and internal factors such as age and genetics negatively affect the effective response to SARS-CoV-2. Additionally, defects in the immune response towards the virus, such as reduced MBL levels and natural antibodies, anti-IFN autoantibodies, and impaired cytotoxic CD8+ cells, may lead to severe infection because of a lack of effective control of viral replication and the spread of propagation from the upper to the lower airway. Simultaneously, a vast release of proinflammatory cytokines and the recruitment of neutrophils, macrophages, and other cell types contribute to uncontrolled systemic inflammation and cytokine storm [33].

We have to mention the thick layer of mucus on the respiratory, gastrointestinal, and reproductive mucosal surfaces in most mammals that contributes to the first-line defense against various infections. Many studies have reported the essential role that mucins play in infectious diseases, including COVID-19[34,35]. In addition, glycans are complex molecules glycans that play a critical role in communication between cells, including adhesion. The extracellular N-terminal domain and intracellular C-terminal domain undergo biochemical changes during bacterial, viral, and parasitic infections by directly influencing both proinflammatory and anti-inflammatory responses[36]. Mucins sense ligands of pathogenic origin and pass the information downstream by activating immunomodulatory pathways. Currently, 22 genes for membrane-bound and secretory mucins have been documented in humans. Recent data suggest that they can be an entry and/or exit for SARS-CoV-2[36]. Furthermore, mucin levels in bronchoalveolar lavage fluid were shown to correlate with cytokine levels, predicting the magnitude of inflammation (*i.e.* cytokine storm), the hallmark of severe COVID-19 and acute respiratory disease syndrome (ARDS). Prognosis and the response to therapy were also influenced by mucin levels[34].

As the virus can enter through the mouth mucosa or the conjunctival surface of the eye, an excellent immune system response would begin with primed and activated immune cells and molecules, including secretory IgA, and SIgA and then spread through the entire mucosa. Moreover, bronchus-associated lymphoid tissue might contribute to the greater resistance to COVID-19 in children, adolescents, and young people compared with older adults[37]. Particular attention should be paid to SIgA, which plays an effective role in protection against various pathogens by neutralization, inhibition of adherence, and agglutination. As SIgA does not activate the classical complement cascade pathway, it is more anti- than proinflammatory[38]. Furthermore, IgA can inhibit IgM or IgG antibody-activated complement. It has been shown that the mucosal immune response with involvement of SIgA begins around 6-10 d after SARS-CoV-2 infection, with the expression of α4β7 integrin mucosal homing receptors and terminal differentiation of B cells into pIgA-secreting plasma cells in NALT[39]. Serum and salivary IgA antibodies against the spike protein of SARS-Cov-2 have also been reported[40]. Moreover, the salivary IgA has been shown to persist for at least 3 mon. Indeed, IgA antibodies against SARS-CoV-2 were found to be higher in the nasal mucosal fluids, tears, and saliva of infected subjects[41,42], IgA-switched plasmablasts that bear the mucosal chemokine receptor CCR10 were increased in the peripheral blood of SARS-CoV-2-infected subjects[42]. Thus, now we have more data on the IgA antibody production in response to SARS-CoV-2 infection.

In addition to MALT, mucosal-associated invariant T cells were also described. They comprise innate-like T cells (*e.g.*, invariant natural killer T, innate lymphoid cells, and γδ T cells) involved in pulmonary mucosal antiviral immunity and tissue protection

and repair after resolving the infection[43]. Pearson *et al*[44] focused on local mucosal responses during viral infection, particularly with SARS-CoV-2 in both lungs and gut. They found that IL-33 and IL-8 were increased in fecal samples of COVID-19 patients due to intestinal involvement[44,45]. Simultaneously, cytokines such as IL-1b, tumor necrosis factor alpha, and IL-6 were found to decrease. IL-7, a critical cytokine for T cell development and survival was also increased during gastrointestinal infection [46]. In addition to the other T cells in the gut mucosa during COVID-19 infection, and enhanced effector function of Th17 cells has also been seen[47]. By secreting many cytokines, they contribute largely to the acute lung injury observed in severe COVID-19 cases. However, their role in mucosal SARS-CoV-2 infection needs to be elucidated.

Considering the route of infection and the relative independence of mucosal and systemic immune responses, one can suggest that appropriate investigations can establish the role of mucosal immune system in SARS-CoV-2 infection for therapy and prevention. In line with this, intranasal COVID-19 vaccines are an additional hope to promote mucosal immunity against the virus, an apparent advantage of other nasal vaccines (*i.e.* influenza)[48]. Moreover, the advantages of such vaccines including generation of both mucosal (SIgA) and circulating (IgG and IgA) antibodies and SARS-specific effector and memory T cell responses have not be seen in conventional vaccines[49].

It is speculated that anti-SIgA antibodies can neutralize and eliminate SARS-CoV-2 in the mucosa without inflammatory consequences. Furthermore, testing for IgA antibodies in nasal and saliva samples might indicate the presence of mucosal immune responses against SARS-CoV-2. Additionally, serum IgA is distinct from locally secreted IgA^+ dimers in plasma cells in the lamina propria of mucosal tissues[50]. The interaction between the virus and the mucosal immune system is shown in Figure 1.

GASTROINTESTINAL INVOLVEMENT DURING SARS-COV-2 INFECTION

Along with its other functions, ACE2 participates in the uptake of amino acids in intestinal epithelial cells, expression of antimicrobial peptides, and gut microbiome ecology[21,51]. As stated above, ACE2 is expressed in almost all human organs, but in varying degrees. Active replication of the SARS-CoV-2 virus has been detected in small-intestine enterocytes isolated from fecal specimens[52]. Other studies showed that the SARS-CoV-2 spike glycoprotein had a 10- to 20-fold higher binding affinity to ACE2 compared with SARS-CoV[53]. ACE2 is highly expressed in the GI[11], and in addition to the small intestine, ACE2 is also highly expressed in the pancreas[54]. Recent studies of single-cell mRNA expression found enriched expression of ACE2 and TMPRSS2 in enterocytes and mucus-producing cells[11,55].

The physiological activities of ACE2 include the absorption of nutrients from digested food. It also maintains osmotic and electrolyte balance across the GI lining epithelium by regulating sodium-dependent amino acid and glucose transporters in the enterocyte brush border[51]. Infectious diarrhea and malabsorption disorders that result from SARS-CoV-2 infection can be explained from a pathophysiological standpoint by the dysregulation of intestinal ion transporters[56]. Studies also suggest dysregulation of these transporters leads to inflammation and GI symptoms[57]. A similar mechanism of enhanced ACE2 expression is known to occur in irritable bowel disease patients who present symptoms similar to those of patients with SARS-CoV-2 [58]. GI cells are potential sites for virus replication of SARS-CoV-2 because of the enriched expression of ACE2 receptors in the mucosal glands and enterocytes[53]. A study using a recombinant strain of SARS-CoV-2 confirmed in situ that the virus could potentially infect and replicate in human intestinal tissue[52]. Once the virus enters the GI cells, it can replicate there, and viral toxin-mediated cell injury can cause gastroenteritis-like symptoms, including diarrhea, nausea, vomiting, and abdominal pain[59].

Infection caused by SARS-CoV-2 is often associated with typical respiratory response and prevalent gastrointestinal symptoms. ACE2 receptors in the GI play a vital role in the genesis of gastrointestinal symptoms. The mechanism underlying the gastrointestinal symptoms may involve damage to the intestinal mucosal barrier and promote the production of inflammatory factors[60]. Studies show that the incidence of gastrointestinal symptoms in SARS-CoV-2 and MERS-CoV infection is more than 20%[61]. Gastrointestinal symptoms may include vomiting, diarrhea, or abdominal pain in the disease's early phases[62]. The cause of the symptoms is an alteration of intestinal permeability and enterocyte dysfunction[63]. One of the first COVID-19 studies included 204 patients from Wuhan, China, infected with the virus with typical respiratory symptoms, many of whom also showed gastrointestinal symptoms, most

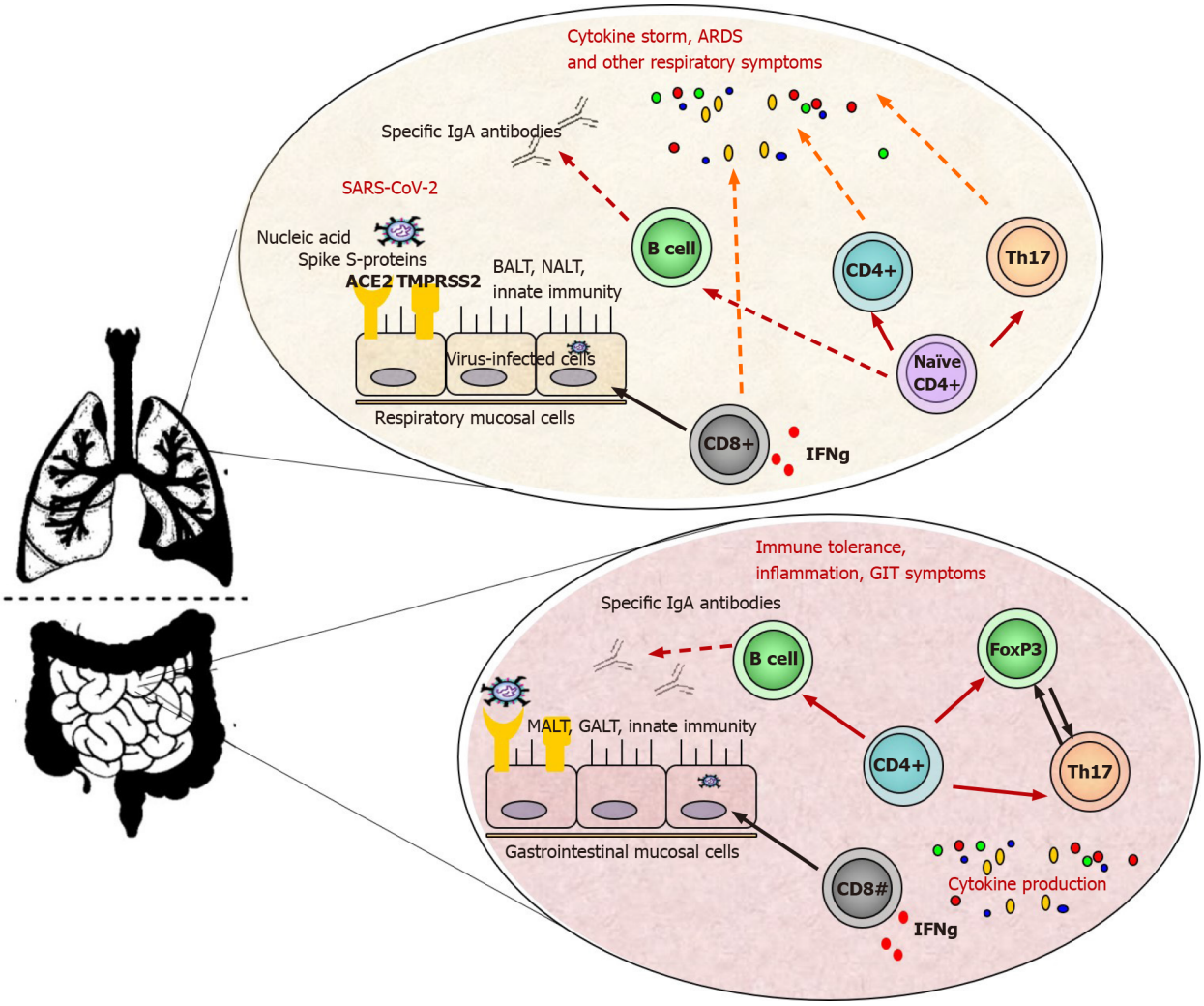


Figure 1 Nasal-, bronchial- and mucosa-associated lymphoid tissue are the first line of defense. Airborne infections usually penetrate the upper airway mucosa, where a higher viral load is found. Nasopharynx-associated lymphoid tissue is involved in the induction of the immune response against the microorganisms by promoting the differentiation and activation of immune cells such as Th1- and Th2 cells, dendritic cells, macrophages, resident microfold M cells, innate lymphoid cells, immunoglobulin (Ig)A-switched B cells, as well as immune mediators and molecules (*i.e.* beta-defensins, galectins, collectins, cytokines). Similar immune processes are also observed in the gut mucosa. However, the expansion of CD4+ T-helper cells, CD8+ cytotoxic T cells, and plasma cells simultaneously with the ongoing innate immune response is critical for virus elimination. Additionally, specific secretory IgA (SIgA) plays an effective role in protection against severe acute respiratory syndrome coronavirus 2 by neutralization, inhibition of adherence, and agglutination. Additionally, SIgA does not activate the classical complement cascade pathway, and thus greater anti- than proinflammatory activity. Innate immune system and some innate receptors (PAMPS, DAMPS) are not shown for simplification of the figure. ACE2: Angiotensin-converting enzyme 2; ARDS: Acute respiratory distress syndrome; BALT: Bronchial-associated lymphoid tissue; GALT: Gut-associated lymphoid tissues; GIT: Gastrointestinal tract. IFN: Interferon; Ig: Immunoglobulin; MALT: Mucosa-associated lymphoid tissue; NALT: Nasopharynx-associated lymphoid tissue; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; TMPRSS2: Transmembrane protease/serine subfamily member 2.

commonly diarrhea. Patients with digestive symptoms have a worse clinical outcome and a longer hospital stay than patients who do not suffer from these symptoms[64].

Although the underlying pathophysiology of gastrointestinal involvement of infection with SARS-CoV-2 is not fully understood, some loss of intestinal barrier integrity and gut microbes is observed. A disruption of intestinal barrier integrity activates innate and adaptive immune cells, which in turn release proinflammatory cytokines into the circulatory system, leading to systemic inflammation[65]. One piece of evidence that SARS-CoV-2 causes an inflammatory response in the gut is elevated levels of fecal calprotectin in patients infected with the virus[66]. Researchers suggest that measuring calprotectin concentrations may play a role in tracking patients infected with SARS-CoV-2. In patients with diarrhea as a symptom, the calprotectin concentrations are elevated, and higher serum IL-6 levels have been reported. It is possible that the disruption of the gut microbiota may be caused by the entry of inflammatory cells, including neutrophils and lymphocytes, into the intestinal mucosa [67]. Studies show that 34% of COVID-19 patients have digestive symptoms, with anorexia and diarrhea being the most common symptoms in adults, while vomiting is

more common in children[68]. Patients with severe COVID-19 have a higher incidence of gastrointestinal symptoms, such as diarrhea and abdominal pain, compared with patients with a mild form of the virus[69]. Nausea and/or vomiting, diarrhea, and loss of appetite are the digestive system's three most common symptoms. Their overall prevalence is around 15% in SARS-CoV-2 infections according to a recent systematic study and meta-analysis involving 6686 patients with GI manifestations. The same study also reported a loss of appetite, ranging from 1% to 79%[69]. The analysis showed that the most common symptom was anorexia (26.8%), but the mechanism remains unclear. The presumption is that widely spread taste and olfactory dysfunctions played a role[70]. Liver injury has also been reported in some patients, with an incidence of 39.6% to 43.4%. The most commonly found elevations are of alanine aminotransferase and aspartate aminotransferase as well as hypoalbuminemia [71].

GUT MICROBIOTA AND COVID-19

The gut microbiota consists of 10^{14} resident microorganisms, including bacteria, viruses, archaea, and fungi[72]. Principally, the gut bacteria in healthy people is dominated by four phyla Actinobacteria, Proteobacteria, Firmicutes, and Bacteroidetes [73]. The gut microbiota has a validated health role through its protective, trophic, and metabolic actions[73]. Loss of healthy commensal bacteria and overgrowth of pathogenic microbes is described as dysbiosis and critical illness. Dysbiosis is related to increased susceptibility to sepsis, multiorgan failure, and nosocomial infections[74]. The development of gut microbiota alternations in COVID-19 depends on SARS-CoV-2 occurrence, the pharmacotherapy of COVID-19, and the disease-associated GI symptoms[75,76].

In a recent study, Xu *et al*[76] described a decrease of beneficial genera, such as *Lactobacillus* and *Bifidobacterium*, in some patients with COVID-19. In another study, Zuo *et al*[77] investigated gut microbiota in 15 SARS-CoV-2 patients by taking fecal samples 2-3 times during their hospital stay. They found reduced commensal bacteria (*Faecalibacterium prausnitzii*, *Eubacterium ventriosum*, *Roseburia*, and *Rachnospiraceae* taxa) and an increased amount of opportunistic pathogens (*Actinomyces viscosus*, *Clostridium hathewayi*, and *Bacteroides nordii*). Furthermore, the abundance of *Clostridium ramosum*, *Coprobacillus*, and *Clostridium hathewayi* was associated with COVID-19 severity[78]. Another exciting study noted that COVID-19 patients had a significantly reduced microbial diversity, a higher abundance of opportunistic bacteria (Streptococcus, Rothia, Veillonella, and Actinomyces), and an increased abundance of beneficial microbes. Additionally, it showed that the microbial signature in the patients was different from those with influenza A and in healthy controls[78]. In summary, the gut microbiota in SARS-CoV-2 infected patients is modified by the reduction of commensal microbes, loss of bacterial diversity, and increased opportunistic pathogens.

The pharmacological therapies used to treat COVID-19 contribute to gut microbiota alterations. A variety of drugs used to treat COVID-19. Among them are chloroquine phosphate, lopinavir, ritonavir, and remdesivir. In cases with pneumonia, broad-spectrum antibiotics are also administered[79]. Antibiotics are well-known modifiers of gut microbiota, and even if short-term use can reduce microbial diversity and cause dysbiosis[80]. Angelakis *et al*[81] demonstrated that gut microbiota alterations were associated with long-term doxycycline and hydroxychloroquine use, leading to significantly decreased amounts of Bacteroidetes, Firmicutes, and *Lactobacillus*. Such changes may also occur in COVID-19 patients, causing gut dysbiosis. Therefore, they may cause the development of gut dysbiosis-related diseases even after improvement of COVID-19 infection. Consequently, it is suggested to screen stool samples taken from recovered patients at least 35 d after the clearance of the virus from the respiratory tract. Before 35 d, SARS-CoV-2 may still be detected in feces[82]. It is also advised to screen the composition and the activity of gut microbiota to describe its balance.

CONCLUSION

As the virus can enter through mouth mucosa, the expectation is that the triggered immune response that occurs somewhere in the mucosa will spread throughout the entire mucosa. This is especially valid for the GI, which may also be a crucial entry or

interaction site of SARS-CoV-2 infection, leading to complex immune activation, digestive symptoms, altered microbiome, development of complications, and eventually to severe COVID-19 and fatal outcome. However, the role of the gut mucosal immune system as the first line of physical and immunological defense is critical. By carrying out appropriate investigations, the mucosal immune system's role in SARS-CoV-2 infection for therapy and prevention can be established. In line with that, COVID-19 vaccines that stimulate mucosal immunity against the virus may have more advantages than other types of vaccines.

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ORIGINAL ARTICLE

Basic Study

Estrogen augmented visceral pain and colonic neuron modulation in a double-hit model of prenatal and adult stress

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Abstract

BACKGROUND

Chronic stress during pregnancy may increase visceral hyperalgesia of offspring in a sex-dependent way. Combining adult stress in offspring will increase this sensitivity. Based on the evidence implicating estrogen in exacerbating visceral hypersensitivity in female rodents in preclinical models, we predicted that chronic prenatal stress (CPS) + chronic adult stress (CAS) will maximize visceral hyperalgesia; and that estrogen plays an important role in colonic hyperalgesia.

AIM

The aim was to illuminate the role of estrogen in colonic hyperalgesia and its underlying mechanisms.

METHODS

We established a CPS plus CAS rodent model in which the balloon was used to distend the colorectum. The single-fiber recording *in vivo* and patch clamp experiments *in vitro* were used to monitor the colonic neuron's activity. The reverse transcription-polymerase chain reaction, western blot, and immunofluorescence were used to study the effects of CPS and CAS on colon primary afferent sensitivity. We used ovariectomy and letrozole to reduce estrogen levels of female rats respectively in order to assess the role of estrogen in female-specific enhanced primary afferent sensitization.

RESULTS

Spontaneous activity and single fiber activity were significantly greater in females than in males. The enhanced sensitization in female rats mainly came from low-threshold neurons. CPS significantly increased single-unit afferent fiber activity in

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L6-S2 dorsal roots in response. Activity was further enhanced by CAS. In addition, the excitability of colon-projecting dorsal root ganglion (DRG) neurons increased in CPS + CAS rats and was associated with a decrease in transient A-type K^+ currents. Compared with ovariectomy, treatment with the aromatase inhibitor letrozole significantly reduced estrogen levels in female rats, confirming the gender difference. Moreover, mice treated with letrozole had decreased colonic DRG neuron excitability. The intrathecal infusion of estrogen increased brain-derived neurotrophic factor (BDNF) protein levels and contributed to the response to visceral pain. Western blotting showed that nerve growth factor protein was upregulated in CPS + CAS mice.

CONCLUSION

This study adds to the evidence that estrogen-dependent sensitization of primary afferent colon neurons is involved in the development of chronic stress-induced visceral hypersensitivity in female rats.

Key Words: Chronic prenatal stress; Estrogen; Visceral pain; Neuronal sensitization; Excitability; Letrozole

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Core Tip: We investigated whether estrogen re-enhanced visceral hyperalgesia in chronic prenatal stress plus chronic adult stress models. After using physical ovariectomy or chemical inhibition with letrozole to reduce estrogen levels, we found that visceral hyperalgesia, colonic afferent neuronal excitability, nerve growth factor and brain-derived neurotrophic factor, and estrogen were all increased. The findings indicate that chronic stress-induced visceral hypersensitivity was estrogen dependent and that the hypersensitivity was mediated by estrogen-dependent sensitization of primary afferent colon neurons. The preclinical models provide key scientific evidence in support of developing gender-based visceral pain management.

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INTRODUCTION

Visceral pain of colonic origin is the most prominent symptom in irritable bowel syndrome (IBS) patients[1]. Female IBS patients report more severe pain that occurs more frequently and with longer episodes than in male patients[1,2]. The ratio of female to male IBS is about 2:1 among patients seen in medical clinics[3]. Moreover, females have a higher prevalence of IBS co-morbidities such as anxiety and depression [4,5] and are more vulnerable to stress-induced exacerbation of IBS symptoms compared with males[3,6,7].

Clinical studies show that early life adverse experiences are risk factors for the development of IBS symptoms, including visceral pain and ongoing chronic stress, especially abdominal pain[8-10]. These factors contribute to the development of visceral hypersensitivity, a key component of the IBS symptom complex and one that may be responsible for symptoms of pain[11,12]. Our previous research found that the female offspring of mothers subjected to chronic prenatal stress (CPS) had a markedly greater visceral sensitivity than their male littermates following challenge by another chronic adult stress (CAS) protocol. A critical molecular event in the development of this female-enhanced visceral hypersensitivity is upregulation of brain-derived neurotrophic factor (BDNF) expression in the lumbar-sacral spinal cord of female CPS + CAS rats[13]. However, the neurophysiological changes underlying the enhanced female-specific visceral hypersensitivity and the role of hormone in the development of stress-induced visceral hypersensitivity are not well understood.

Visceral hypersensitivity in IBS involves abnormal changes in neurophysiology throughout the brain-gut axis. In IBS, there is evidence for sensitization of primary afferents to jejunal distention and electrical stimulation[14], and there is evidence for increased sensitivity of lumbar splanchnic afferents[15,16]. In animal models of either early life adverse events or adult stress-induced visceral hypersensitivity[17], there is evidence of colon primary afferent sensitization. However, the studies were performed in male rodents. Therefore, in this study, we established a CPS and CAS rodent model to analyze the impact on female colon afferent neuron function and the role of estrogen. Our hypothesis was that female CPS offspring subjected to chronic stress as adults would exhibit greater colonic dorsal root ganglion (DRG) neuron sensitization compared with their male littermates, and that the enhanced visceral sensitization and primary afferent sensitization in females was estrogen dependent.

MATERIALS AND METHODS

Animals

The Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston, TX approved all animal procedures. Experiments were performed on pregnant Sprague Dawley rats and their 8-wk-old to 16-wk-old male and female offspring. Rats were housed individual cages with access to food and water in a room with controlled conditions (22 ± 2 °C, relative humidity of 50% ± 5%), and a 12 h light/12 h dark cycle.

CPS and CAS models

Pregnant dams were subjected to a CPS protocol that consisted of a random sequence of twice-daily applications of one of three stress sessions, a 1-h water-avoidance, 45-min cold-restraint, or a 20-min forced swim starting on day 6 and continuing until delivery on day 21. Male and female offspring of the stressed dams were designated CPS rats. Control dams received sham stress and their offspring were designated control rats. As adults at 8-16 wk of age, control and prenatally stressed offspring were challenged by the same CAS protocol for 9 d. Ovariectomy (OVX) or sham surgery was performed on female prenatal-stress offspring on day 56. Daily letrozole treatment was initiated on day 49, 2 wk prior to initiation of adult stress. Treatment was continued through the stress protocol. A schematic diagram of the study procedures is shown in Figure 1A.

Rat treatment

Before OVX or letrozole treatment, vaginal smears were used to identify the estrus cycle phase. OVX or sham surgery was performed on female prenatal-stress offspring on day 56. The aromatase inhibitor letrozole [4,4′-(1H-1,2,4-triazol-l-yl-methylene)-bis-benzonitrile], (Novartis) 1.0 mg/kg was orally administered in the experimental group and vehicle (hydroxypropyl cellulose 0.3% in water) was given in the control group once daily for 14 d. Direct transcutaneous intrathecal injections of estrogen and letrozole were performed as described by Mestre *et al*[18].

In vivo single fiber recording of L6-S2 DRG rootlets

Multiunit afferent discharges were recorded from the distal ends of L6-S2 dorsal rootlets decentralized close to their entry into the spinal cord. A bundle of multiunit fibers was distinguished into 2-6 single units off-line using wave mark template matching in Spike 2 software that differentiates spikes by shape and amplitude. Colonic afferent fibers were identified by their response to graded colorectal distention (CRD). A balloon was used to distend the colorectum. Isoflurane, 2.5%, followed by 50 mg/kg intraperitoneal sodium pentobarbital induced general anesthesia that was maintained by infusing a mixture of pentobarbital sodium + pancuronium bromide + saline by intravenous infusion through the tail vein. The adequacy of anesthesia was confirmed by the absence of corneal and pupillary reflexes and stability of the end-tidal CO_2 level. A tracheotomy tube connected to a ventilator system provided a mixture of room air and oxygen. Expired CO_2 was monitored and maintained at 3.5%. Body temperature was monitored and maintained at 37 °C by a servo-controlled heating blanket. A laminectomy from T12 to S2 exposed the spinal cord. The head was stabilized in a stereotaxic frame.

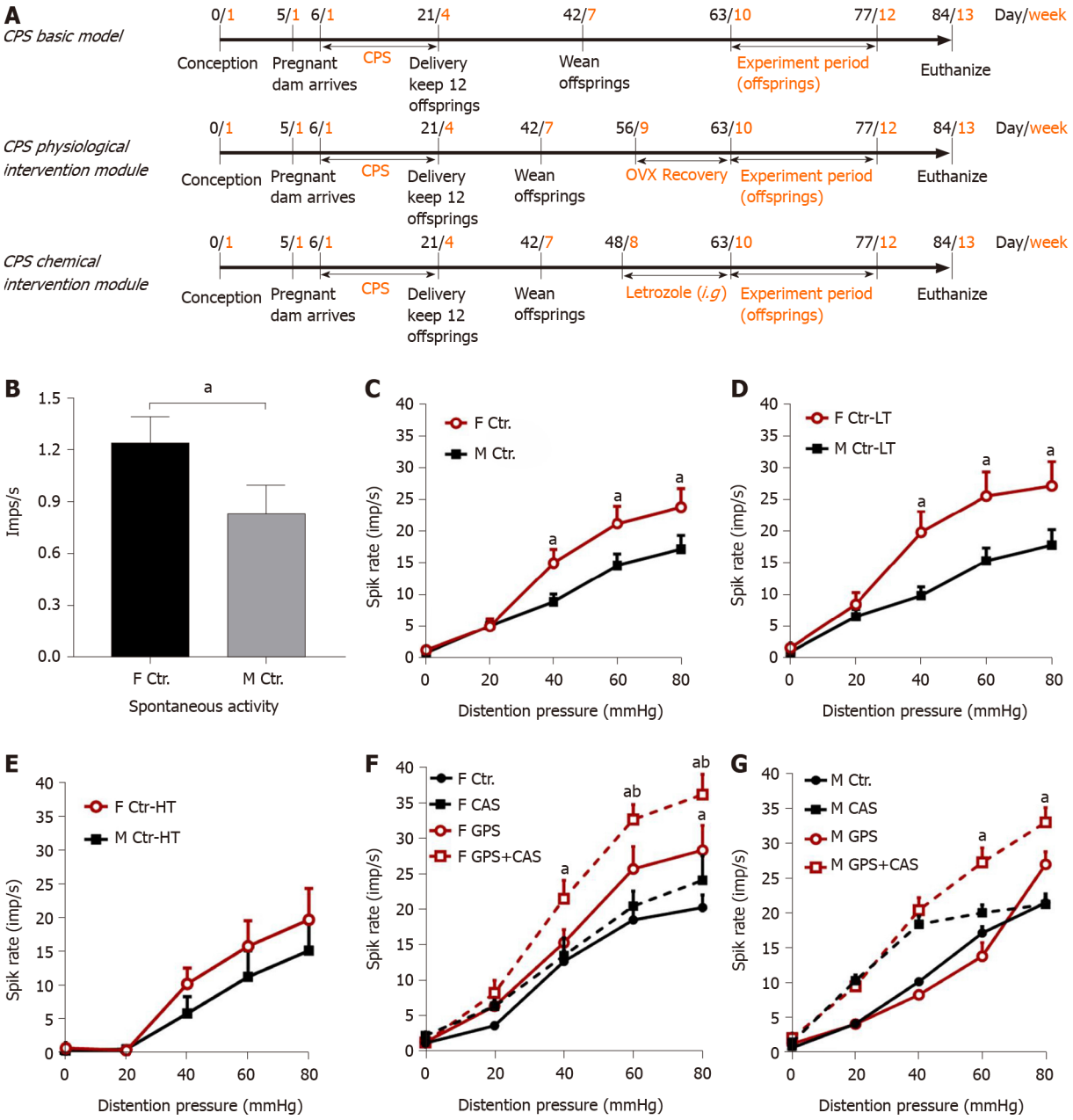


Figure 1 Primary afferent responses to colorectal distention. A: Chronic prenatal stress (CPS) plus chronic adult stress (CAS) model. Pregnant dams were subjected to prenatal stress from on day 11 of gestation. Ovariectomy (OVX) or sham surgery was performed on female prenatal-stress offspring on day 56. Daily Letrozole was initiated on day 49, 2 wk prior to initiation of adult stress. Treatment was continued through the stress protocol; B: Spontaneous activity (SA) of single afferent units in male and female control rats (n = 70 fibers in 6 rats in each group, t-test, $^{a}P < 0.05$); C: Average response to graded colorectal distention (CRD) of 56 afferent fibers in 6 male and 70 afferent fibers in 6 female control rats; two-way analysis of variance (ANOVA; $^{a}P < 0.05$ *vs* the same pressure male group); D: Responses of low-threshold (LT) fibers to CRD in 42 fibers in 6 male rats and 40 fibers in 6 female control rats (ANOVA, $^{a}P < 0.05$ *vs* the same pressure male group); E: Responses of high-threshold (HT) afferent fibers to CRD in 14 fibers in 6 male and 29 fibers in 6 female control rats; F: Effects of CAS on afferent fiber responses to CRD from 59 fibers in 6 control and 99 fibers in 6 CPS female rats; (two-way ANOVA, $^{a}P < 0.05$ *vs* the same pressure control group, $^{b}P < 0.05$ *vs* the same pressure CPS group); G: Effects of CAS on afferent fiber responses to CRD in control and CPS male rats (n = 6 rats, 57 fibers for control and 95 fibers for CPS female group; two-way ANOVA, $^{a}P < 0.05$ *vs* the same pressure-control group).

In vitro patch clamp recordings in colonic DRG neurons

Retrograde fluorescence label injections: Labeling of colon-projecting DRG neurons was performed as previously described[13]. Under general 2% isoflurane anesthesia, the lipid soluble fluorescent dye, 1,1′-dioleyl-3,3,3′,3′-tetramethylindocarbocyanine methane-sulfonate (9-DiI, Invitrogen, Carlsbad, CA) was injected (50 mg/mL) into the muscularis externa on the exposed distal colon in 8 to 10 sites (2 μL each site). To prevent leakage, the needle was kept in place for 1 min following each injection.

Dissociation and culture of DRG neurons: Rats were deeply anesthetized with isoflurane followed by decapitation. Lumbosacral (L6–S2) DRGs were collected in ice

cold and oxygenated dissecting solution, containing (in mM) 130 NaCl, 5 KCl, 2 KH_2PO_4, 1.5 $CaCl_2$, 6 $MgSO_4$, 10 glucose, and 10 HEPES, pH 7.2 (305 mOsm). After removal of the connective tissue, the ganglia were transferred to a 5 mL dissecting solution containing collagenase D (1.8 mg/mL; Roche) and trypsin (1.0 mg/mL; Sigma, St Louis, MO), and incubated for 1.5 h at 34.5 °C. DRGs were then taken from the enzyme solution, washed, and put in 0.5-2 mL of the dissecting solution containing DNase (0.5 mg/mL; Sigma). Cells were subsequently dissociated by gentle trituration 10 to 15 times with fire-polished glass pipettes and placed on acid-cleaned glass coverslips. The dissociated DRG neurons were kept in 1 mL DMEM (with 10% FBS) in an incubator (95% O_2/5% CO_2) at 37 °C overnight.

Whole-cell patch clamp recordings from dissociated DRG neurons: Before each experiment, a glass coverslip with DRG neurons was transferred to a recording chamber perfused (1.5 mL/min) with external solution containing (10 mM): 130 NaCl, 5 KCl, 2 KH_2PO_4, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose, pH adjusted to pH 7.4 with NaOH (300 mOsm) at room temperature. Recording pipettes, pulled from borosilicate glass tubing, with resistance of 1-5 MΩ, were filled with solution containing (in mM): 100 $KMeSO_3$, 40 KCl, and 10 HEPES, pH 7.25 adjusted with KOH (290 mOsm). DiI-labeled neurons were identified by fluorescence microscopy. Whole-cell currents and voltage were recorded from DiI-labeled neurons using a Dagan 3911 patch clamp amplifier. Data were acquired and analyzed by pCLAMP 9.2 (Molecular Devices, Sunnyvale, CA). The currents were filtered at 2–5 kHz and sampled at 50 or 100 s *per* point. While still under voltage clamp, the Clampex Membrane Test program (Molecular Devices) was used to determine membrane capacitance (Cm) and membrane resistance (Rm), during a 10 ms, 5 mV depolarizing pulse form a holding potential of −60 mV. The configuration was then switched to current clamp (0 pA) to determine other electrophysiological properties. After stabilizing for 2–3 min, the resting membrane potential was measured. The minimum acceptable resting membrane potential was −40 mV. Spontaneous activity (SA) was then recorded over two 30 s periods separated by 60 s without recording, as described by Bedi *et al*[19].

Transient A-type K^+ current (I_A) recording method in patch studies: To record voltage-gated K^+ current (Kv), Na^+ in control external solution was replaced with equimolar choline and the Ca^{2+} concentration was reduced to 0.03 mM to suppress Ca^{2+} currents and to prevent Ca^{2+} channels becoming Na^+ conducting. The reduced external Ca^{2+} would also be expected to suppress Ca^{2+}-activated K^+ currents. The current traces of Kv in DRG neurons were measured at different holding potentials. The membrane potential was held at −100 mV and voltage steps were from −40 to +30 mV to record the total Kv. The membrane potential was held at −50 mV to record the sustained Kv. The I_A currents were calculated by subtracting the sustained current from the total current. The current density (in pA/pF) was calculated by dividing the current amplitude by cell membrane capacitance.

Real time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using RNeasy Mini Kits (QIAGEN, Valencia, CA). One microgram of total RNA was reverse-transcribed using the SuperScript™ III First-Strand Synthesis System. PCR assays were performed on a StepOnePlus thermal cycler with 18 s as the normalizer using Applied Biosystems primer/probe set Rn02531967_s1 directed against the translated exon IX. Fold-change relative to control was calculated using the ΔΔCt method (Applied Biosystems).

Western blot

Samples were lysed in RIPA buffer containing protease inhibitor cocktail and phenylmethanesulfonyl fluoride. Lysates were incubated for 30 min on ice and then centrifuged at 10 000 × *g* for 10 min at 4 °C. The protein concentration in the supernatant was determined using bicinchoninic acid (BCA) assay kits with bovine serum albumin as a standard. Equal amounts of protein (30 μg per lane) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes (Bio-Rad, United States). The membranes were blocked in Li-Cor blocking buffer for 1 h at room temperature and then incubated with primary antibodies. BDNF antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) was used at 1:200 dilution; nerve growth factor (NGF) antibody (Abcam, MA) was used at 1:1000 dilution; β-actin antibody (Sigma Aldrich, St Louis, MO) was used at 1:5000 dilution. The secondary antibodies were donkey anti-rabbit Alexa Fluor 680 (Invitrogen) and goat anti-mouse IRDye 800 (Rockland). Images were

acquired and band intensities measured using a Li-Cor Odyssey system (Li-Cor, Lincoln, NE).

Immunofluorescence

Frozen sections of colon tissue from control, CAS, CPS and CPS + CAS female rats were mounted on glass slides, and rehydrated in phosphate buffered saline at room temperature. The slides were treated for antigen retrieval and blocked with 10% normal goat serum diluted in 0.3% phosphate buffered saline-Triton for 1 h, and then incubated with NGF primary antibody in antibody diluent (Renoir Red, Biocare Medical, Concord, CA) at 4 °C overnight. The slides were exposed to fluorescent dye-conjugated secondary antibody for 2 h at room temperature, counterstained with 4',6-diamidino-2-phenylindole and coverslipped. Images were taken in fluorescence mode on an Olympus laser scanning confocal microscope and the average signal intensity was calculated by the bundled software.

Serum estradiol and norepinephrine levels

Serum estradiol, adrenocorticotropic hormone (ACTH), and norepinephrine levels were measured using specific enzyme-linked immunosorbent assay kits for each analyte (CSB-E05110r, CSB-E06875r, CSB-E07022, Cusabio Bioteck CO., United States) following the manufacturer's instructions.

Data analysis

Single fiber responses (impulses/second) to CRD were calculated by subtracting SA from the mean 30 s maximal activity during distension. Fibers were considered responsive if CRD increased their activity to 30% greater than the baseline value. Mechanosensitive single units were classified as high threshold (> 20 mmHg) or low threshold (≤ 20 mmHg) on the basis of their response threshold and profile during CRD. Single fiber activity data were analyzed by analysis of variance with repeated measures; CRD intensity was the repeated factor and the experimental group was the between-group factor. If significant main effects were present, the individual means were compared using the Fisher post-hoc test. All authors had access to the study data and reviewed and approved the final manuscript.

RESULTS

Effects of CPS plus CAS on primary afferent responses to CRD in male and female rats

The basal activity of a spinal afferent fiber was defined as the average number of action potentials per second (impulses/sec) in the 60 s period before the onset of a distention stimulus. In male controls, 66% of the afferent fibers under study displayed SA and SA was significantly higher in female controls than in male controls (0.71 ± 0.21 *vs* 1.24 ± 0.20 imp/sec; Figure 1B). The average single fiber activity in response to CRD was significantly higher in female control rats compared with male controls (Figure 1C). We found that the enhanced sensitization in female rats mainly came from the low-threshold fibers (Figure 1D and E).

To assess the effects of CPS + CAS on colon afferent fiber activities, we compared average single colon afferent fiber activities projecting from dorsal roots S1-L6 in response to CRD in male and female control, CPS, control + CAS and CPS + CAS rats recorded approximately 24 h after the last stressor. In females, CPS significantly increased single-unit afferent activity in response to CRD *vs* control female rats (Figure 1F). CAS alone enhanced single-unit activity compared with control. The increase in average afferent responses after CAS in prenatally stressed female rats (44.0%) was significantly greater than the increase in female control rats (39.3%). In males, CPS had no significant effect on primary afferent responses (Figure 1G). When we compared males to females within each experimental group, we found that the average single fiber activity was significantly higher in female compared with male CPS + CAS rats (Figure 1F, G). The increased activity may contribute to the enhanced female visceral hypersensitivity previously reported in this model. Average single-fiber activities were significantly greater in control and CPS and CAS females than in their corresponding male experimental groups (Figure 1F and G). Both CAS and CPS + CAS rats had significantly increased primary afferent responses compared with control and CPS rats. Thus, our CPS and CAS protocols sensitized colon-projecting primary afferent fibers, with the greatest effects produced by the combination of CPS +

CAS in both males and females.

Increase in excitability of colon-projecting lumbosacral DRG neurons in female CPS + CAS rats

To elucidate the electrophysiological basis of enhanced stress-induced primary afferent activity in female rats, we performed patch clamp studies on acutely dissociated retrograde-labeled colon-projecting neurons from the L6-S2 DRGs in control, prenatal stress, adult stress only, and CPS + CAS female rats isolated 24 h after the last adult stressor (Figure 2A). Input resistance (Figure 2B) and rheobase (Figure 2C) were significantly decreased in neurons from CPS + CAS rats compared with the other three groups. The number of action potentials elicited at either 2 × or 3 × the rheobase were significantly greater in adult stress and CPS + CAS neurons compared with control and to CPS neurons (Figure 2D, E). CAS significantly increased action potential overshoot with or without CPS (Figure 2F), but it did not significantly alter other electrophysiological characteristics, such as number of spontaneous spikes, membrane capacitance (pF), resting membrane potential, cell diameter, time constant, and DRG neuron action potential amplitude and duration (Table 1).

The percentage of neurons with SA in was significantly greater in CPS + CAS rats than in control or CPS only rats (Figure 2G). Under voltage clamp conditions (Figure 2H), neurons from female CPS + CAS, CAS, CPS and control groups had I_A and sustained outward rectifier K^+ currents (I_K). Compared with the other three groups, DRG neurons from CPS + CAS rats had significantly reduced average I_A ($P < 0.05$). The average I_K density was decreased but the change was not significant.

Effects of CPS and/or CAS on plasma estrogen concentration

We did a vaginal smear test to identify the estrus cycle phases by identifying the vaginal cytological cell types. Estrogen concentration was significantly higher in the CPS proestrus/estrus phase compared with control diestrus, control proestrus/estrus, and CPS diestrus proestrus ($P < 0.05$; Figure 3A). Comparison of the plasma estrogen concentrations in control, CAS, CPS, CPS + CAS showed that CPS significantly increased plasma estrogen levels compared with the control rats and that CAS increased plasma estrogen level compared with the control and CPS rats (Figure 3B).

To determine whether estrogen contributed to stress-induced visceral hypersensitivity in prenatal stressed females, we reduced plasma estrogen levels by either OVX or letrozole treatment. OVX significantly lowered serum estradiol levels before and after CAS (Figure 3C). Treatment was continued throughout CAS. After treatment with letrozole, serum estradiol levels were significantly reduced (Figure 3D). To study the effects of gender and stress on norepinephrine and ACTH levels, we measured plasma norepinephrine levels in female rats from all four experimental groups. CAS alone significantly increased plasma norepinephrine levels compared with both the controls and with CPS alone (Figure 3E) Plasma norepinephrine levels were significantly increased in CPS + CAS rats compared with CAS alone as well as with controls and CPS. Plasma ACTH levels were significantly increased in CPS + CAS rats compared with controls. (Figure 3F).

Effects of Letrozole treatment on colon DRG neuron excitability

We performed patch clamp experiments on acutely isolated retrograde-labeled DRG neurons from CPS + CAS females with or without letrozole treatment 24 h after the last adult stressor. Letrozole treatment significantly increased rheobase (Figure 4A), and significantly reduced input resistance (Figure 4B). Action potential overshoot (Figure 4C) and the number of action potentials elicited by a current injection at either 2 × or 3 × rheobase were significantly reduced by letrozole treatment (Figure 4D). Other electrophysiological properties were not significantly altered (Table 2). We also recorded electromyographic activity to determine whether the reduction in visceral sensitivity in female CPS + CAS rats caused by OVX or systemic letrozole treatment reduced visceromotor responses. The findings demonstrated a significant decrease in excitability of colon-projecting L6-S2 neurons.

Spinal cord BDNF levels regulated by estrogen

To investigate the effect of estrogen on BDNF expression, we measured BDNF mRNA and protein levels in the lumbar-sacral spinal cords of OVX and Sham CPS + CAS female rats. Systemic estradiol administration to naïve cycling females produced significant increases in plasma estrogen (Figure 5A), lumbar-sacral spinal cord BDNF mRNA (Figure 5B), and protein (Figure 5C). We also measured BDNF mRNA and protein levels in the lumbar-sacral spinal cords of OVX and Sham CPS + CAS female

Table 1 Electrophysiological characteristics of colon related DRG neuron

Classification	Ctr., *n* = 36	CAS, *n* = 34	CPS, *n* = 29	CAS + CPS, *n* = 45
Spontaneous spike number	7.4 ± 4.0	16.9 ± 6.6	11.7 ± 4.8	44.8 ± 14.6[c]
Membrane capacitance (pF)	72.1 ± 4.9	95.4 ± 5.8	85.1 ± 6.2	93.8 ± 8.9[a]
Action potential threshold (mV)	-27.1 ± 2.5	-29.2 ± 1.8	-34.6 ± 1.6	-38.6 ± 1.4[c]
Resting membrane potential (mV)	-60.1 ± 1.7	-50.1 ± 1.3	-53.8 ± 1.5	-56.4 ± 1.3
Cell diameter (μm)	32 ± 0.9	29 ± 0.6	31 ± 0.8	31 ± 0.6
Time constant (μm)	545.5 ± 51.1	737.7 ± 70.4	595.9 ± 54.1	535.3 ± 42.5
Action potential amplitude (mV)	79.0 ± 4.7	80.2 ± 3.9	77.4 ± 4.2	85.6 ± 3.7
Duration (ms)	8.38 ± 0.97	11.2 ± 0.95	12.3 ± 1.83	8.89 ± 0.60

Values means ± standard error.
[a]$P < 0.05$;
[c]$P < 0.001$ *vs* control group. CAS: Chronic adult stress; CPS: Chronic prenatal stress.

Table 2 Electrophysiological characteristics of colon related DRG neuron after Letrozole treatment

Classification	Veh. + CAS + CPS, *n* = 60	Let + CAS + CPS, *n* = 27
Spontaneous spike number	34.6 ± 11.3	8.04 ± 4.62[c]
Membrane capacitance (pF)	86.8 ± 7.1	83.7 ± 6.7
Action potential threshold (mV)	-34.6 ± 1.6	-27.6 ± 2.1[a]
Resting membrane potential (mV)	-54.9 ± 1.3	-54.6 ± 2.2
Cell diameter (μm)	31 ± 0.5	27 ± 1.6
Time constant (um)	502.7 ± 34.5	454.0 ± 36.6
Action potential amplitude (mV)	90.9 ± 3.7	89.8 ± 7.2
Duration (ms)	8.67 ± 0.63	10.70 ± 2.22

Values are means ± standard error.
[a]$P < 0.05$;
[c]$P < 0.001$ *vs* control group. CAS: Chronic adult stress; CPS: Chronic prenatal stress.

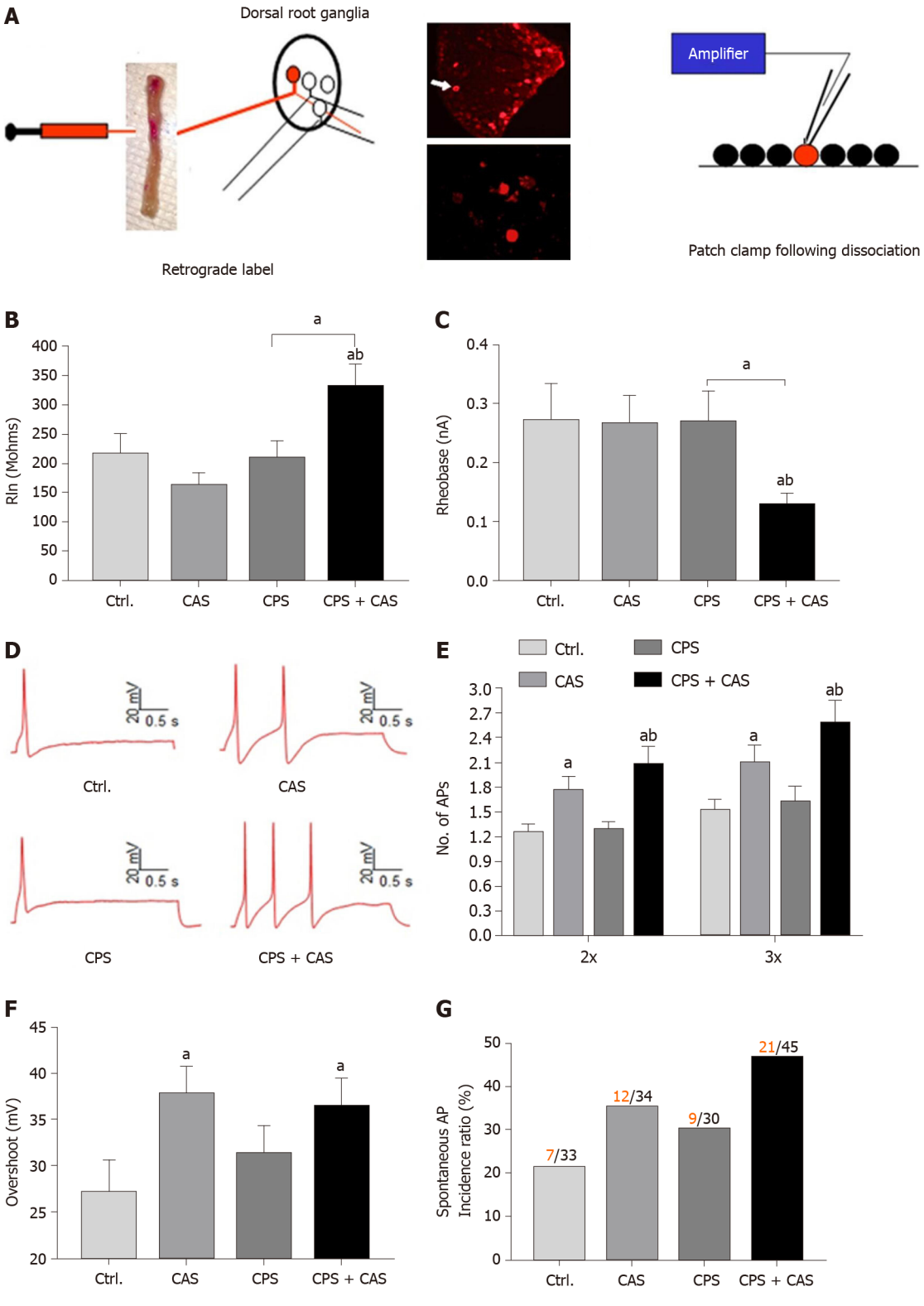
rats. BDNF mRNA and protein expression were significantly suppressed by OVX compared with sham rats. Another experiment showed that intrathecal infusion of estrogen into naïve female rats significantly increased BDNF protein levels, which proved that estrogen reversed the experimental results and contributed to the response to visceral pain[13].

Peripheral NGF level increased in CPS + CAS female rats

We examined NGF expression in the colons of females from all four experimental groups by immunohistochemistry (Figure 6A). Morphometric analysis showed that CAS and CPS + CAS significantly increased NGF levels in the colon wall, with the increase in CPS + CAS significantly greater that of CAS alone (Figure 6B). Western blotting showed that NGF protein was significantly upregulated in CPS + CAS rats compared with controls (Figure 6C).

DISCUSSION

Enhanced CPS-induced visceral hypersensitivity in female rats was associated with an increase in the responses of lumbosacral nerve fibers to CRD in both male and female offspring. These findings are further supported by data showing increased excitability of colon-projecting DRG neurons from females in patch clamp studies. The magnitude of the sensitization was the greatest in female CPS + CAS rats, suggesting that it made


A
Dorsal root ganglia
Retrograde label
Amplifier
Patch clamp following dissociation
B
Rin (Mohms)
Ctrl.
CAS
CPS
CPS + CAS
C
Rheobase (nA)
D
20 mV
0.5 s
E
No. of APs
2x
3x
F
Overshoot (mV)
G
Spontaneous AP Incidence ratio (%)
7/33
12/34
9/30
21/45

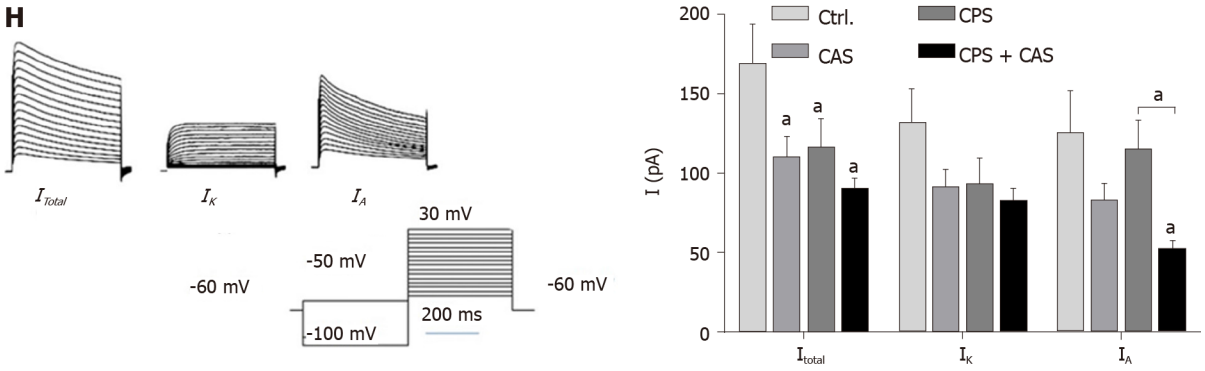


Figure 2 Patch clamp recording in colonic dorsal root ganglion neurons from female rats. A: Patch clamp process of cell labeling. Under isoflurane anesthesia, the lipid soluble fluorescent dye 9-DiI was injected into muscularis externa of the exposed distal colon (left figure). Lumbosacral (L6–S2) dorsal root ganglions (upper photograph) were isolated and DiI-labeled neurons were identified by fluorescence microscopy (lower photograph). Electrophysiological properties of each neuron were measured using whole-cell current and voltage clamp protocols (right figure); B: Rheobase from all four experimental groups (n = 5 rats, 45 cells in each group, one-way ANOVA, $^aP < 0.05$ *vs* control or $^bP < 0.05$ *vs* CAS); C: Representative action potentials (APs) elicited by current injection at 2 × the rheobase in neurons from control, chronic adult stress (CAS), chronic prenatal stress (CPS) and CPS + CAS female rats; D: Membrane input resistance from all four groups (n = 5 rats, 45 cells in each group, one-way ANOVA, $^aP < 0.05$ *vs* control; $^bP < 0.05$ *vs* CPS); E: Number of APs elicited by current injection at either 2 × and 3 × the rheobase in all four experimental groups (two-way ANOVA, $^aP < 0.05$ *vs* control; $^bP < 0.05$ *vs* CPS); F: AP overshoot recorded from all four experimental groups ($^aP < 0.05$ *vs* control); G: The proportion of neurons from each experimental group exhibiting spontaneous APs. Red numbers represent spontaneous AP firing cells; black numbers represent total cells; H: Representative total, I_K and I_A current tracings and average values of potassium currents: I_{total}, I_K and I_A are shown in female CPS + CAS, CAS, CPS (n = 15 neurons, from 5 rats in each group), and control groups (n = 12 neurons from 5 rats); two-way ANOVA, $^aP < 0.05$ *vs* each control group.

a major contribution to the observed enhanced female visceral hypersensitivity in our model.

Chronic stress is known to increase the excitability of colon-projecting DRG neurons in rats and mice. In adult male Sprague Dawley rats, colon DRG neuron sensitization was shown to be driven by increases in NGF expression in the colon muscularis externa[13]. In our model, we also observed a significant increase in colon NGF, but its potential role in primary afferent sensitization and visceral hypersensitivity was not investigated.. Other studies in male mice showed that stress, in the form of water avoidance, significantly increased the excitability of colon-projecting DRG neurons and that the combined activity of the stress mediators corticosterone and norepinephrine increased DRG neuron excitability in vitro[20,21]. We previously found significant increases in the serum levels of norepinephrine in CPS + CAS females. However, daily systemic treatment with adrenergic antagonists during the adult stress protocol failed to reduce visceral hypersensitivity in female neonatal + adult stress rats [13] suggesting that norepinephrine did not play a major role in the acquisition of enhanced female visceral hypersensitivity or primary afferent sensitization in our model.

When we tested on lumbar-sacral afferent fibers and dissociated neurons in patch clamp studies, we found significant decreases in transient potassium I_A currents in neurons isolated from CPS + CAS females compared with the other three experimental groups. Declines in A-type Kv currents in DRG neurons have been associated with persistent pain in multiple chronic pain models[22]. Whether the decline was caused by changes in channel properties or expression was not investigated in this study. However, another study demonstrated that estrogen significantly shifted the activation curve of I_A currents in the hyperpolarizing direction and that estrogen inhibited Kv (+) channels in mouse DRG neurons through a membrane ER-activated nongenomic pathway[23].

Our results showed that the excitability of colon-projecting neurons in CPS + CAS females was significantly reduced by systemic letrozole treatment, suggesting that estrogen contributed to the sensitization process. Previous studies show that estrogen receptors expressed on primary afferent neurons contributed to enhanced sensitivity in various pain models[24-26]. One study found no decline in the responses of colon-projecting nerve fibers to CRD following OVX and found no detectable estrogen receptor alpha immunoreactivity in colon-projecting DRG neurons[27]. The reasons for the differing results are not clear, but local production of estrogen in DRG neurons could be sufficient to sustain sensitization.

NGF and its receptors play important roles in the mechanism of visceral pain and hyperalgesia in women. For example, endometriosis is estrogen dependent and is commonly diagnosed. The main symptoms are various types of pelvic pain that have a

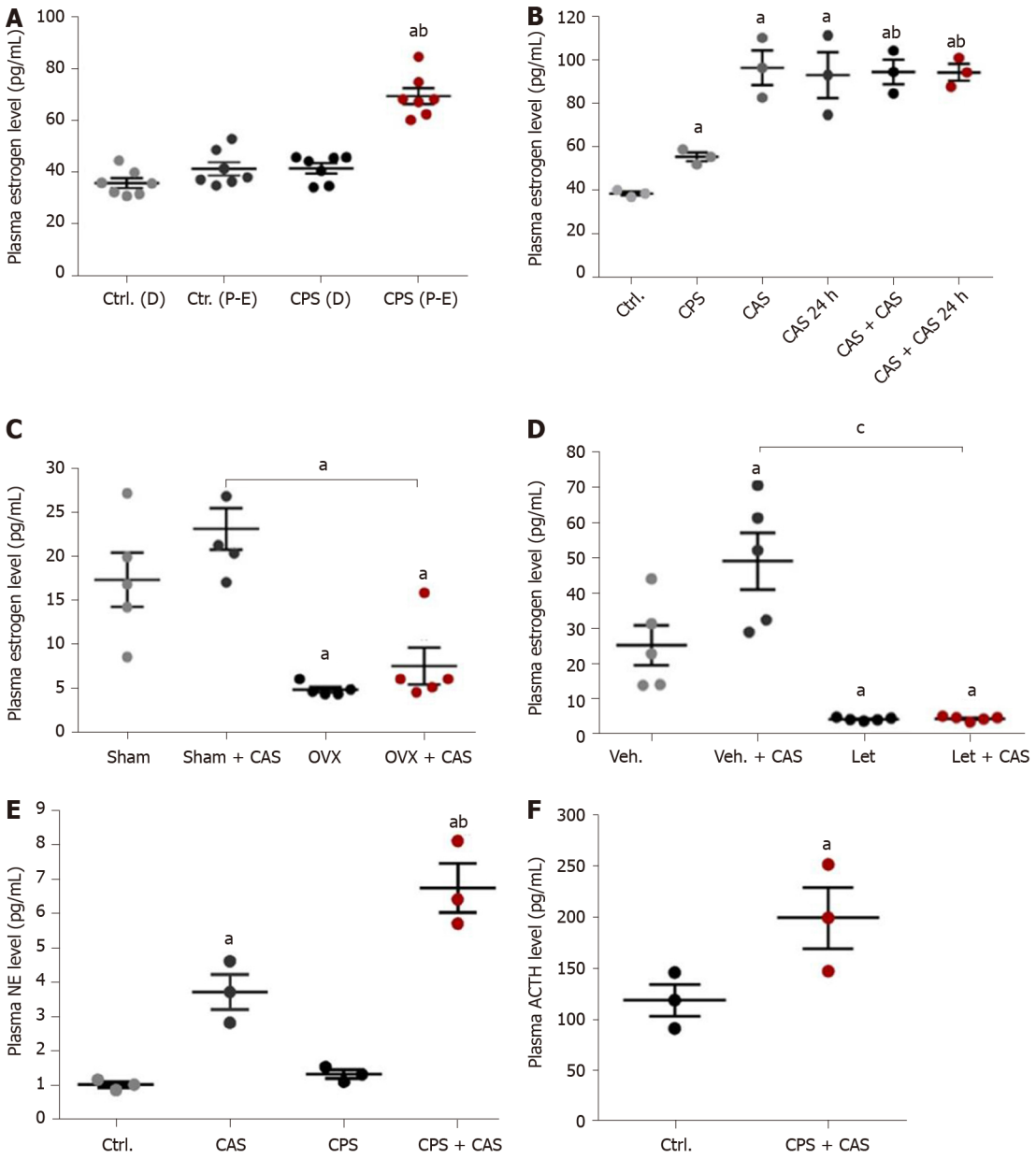


Figure 3 Effects of chronic prenatal stress, chronic adult stress, ovariectomy, and letrozole treatment on plasma estrogen levels in female rats. A: Plasma estrogen level in control and chronic prenatal stress (CPS) rats by estrus cycle phase (n = 8 rats, one-way ANOVA, $^{a}P < 0.05$ *vs* control proestrus/estrus (P-E) phase; $^{b}P < 0.05$ *vs* CPS diestrus (D) phase); B: Plasma estrogen levels increased in CPS rats and following chronic adult stress (CAS) 24 h after the last adult stressor (n = 8 rats, one-way ANOVA, $^{a}P < 0.05$ *vs* control; $^{b}P < 0.05$ *vs* CPS); C: Ovariectomy (OVX) significantly reduced CPS female rat plasma estrogen levels before and after CAS (n = 5 rats, one-way ANOVA, $^{a}P < 0.05$ *vs* sham group); D: Letrozole treatment significantly reduced CPS female rat plasma estrogen levels before or after CAS (n = 5 rats, one-way ANOVA, $^{a}P < 0.05$ *vs* vehicle group; $^{c}P < 0.0001$); E: Plasma norepinephrine levels from control, CAS, CPS and CPS + CAS group female rats (n = 5 rats, one-way ANOVA, $^{a}P < 0.05$ *vs* control; $^{b}P < 0.05$ *vs* CPS); F: Plasma adrenocorticotropic hormone (ACTH) levels from control and CPS + CAS group female rats (n = 5 rats, t-test, $^{a}P < 0.05$ *vs* control).

serious effect on physical and mental health, but the mechanisms of abdominal pain are still unclear. Studies have shown NGF to be an inflammatory mediator and modulator of pain in adulthood[28].

CONCLUSION

In this study, we examined the sex differences and effects of estrogen on the acquisition of enhanced visceral hypersensitivity in the offspring of rats in a model of prenatal and adult stress as shown in Figure 7. Our study shows that estrogen acted in the spinal cord and the primary afferent neurons to enhance visceral nociception. Acute blockade of the endogenous synthesis of estrogens in rat spinal cord

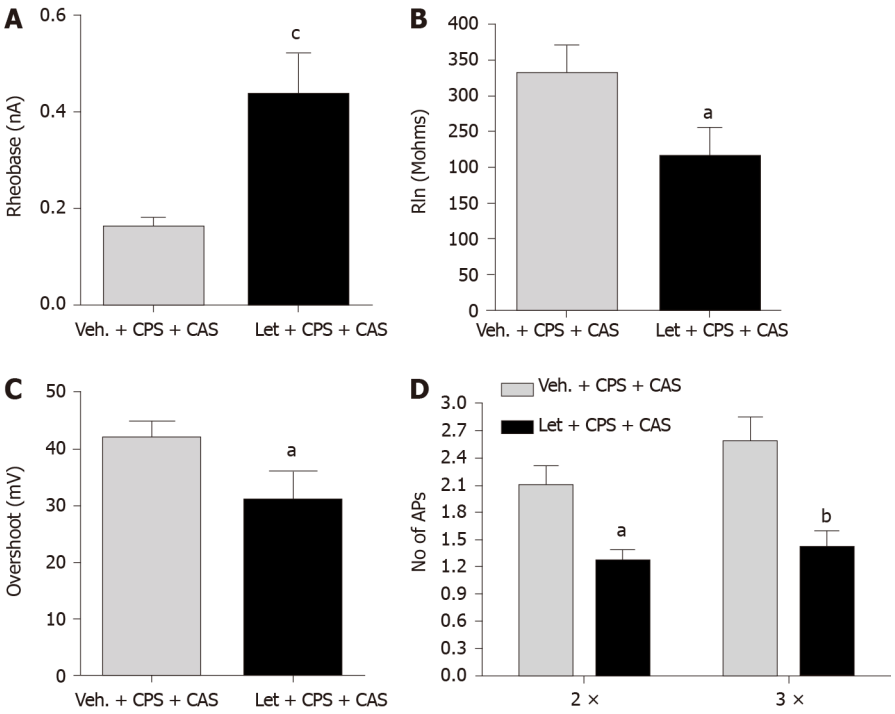


Figure 4 Effects of Letrozole treatment on colon dorsal root ganglion neuron excitability. A: Rheobase (n = 45 cells in 6 rats in each group, t-test, $^{c}P < 0.001$ *vs* Veh. + chronic adult stress [CAS] + chronic prenatal stress [CPS]); B: Membrane input resistance (Rin) (t-test, $^{a}P < 0.05$); C: Action potential (AP) overshoot (t-test, $^{a}P < 0.05$); D: Number of APs elicited by current injection at 2 × and 3 × rheobase (two-way ANOVA, $^{a}P < 0.05$; $^{b}P < 0.01$).

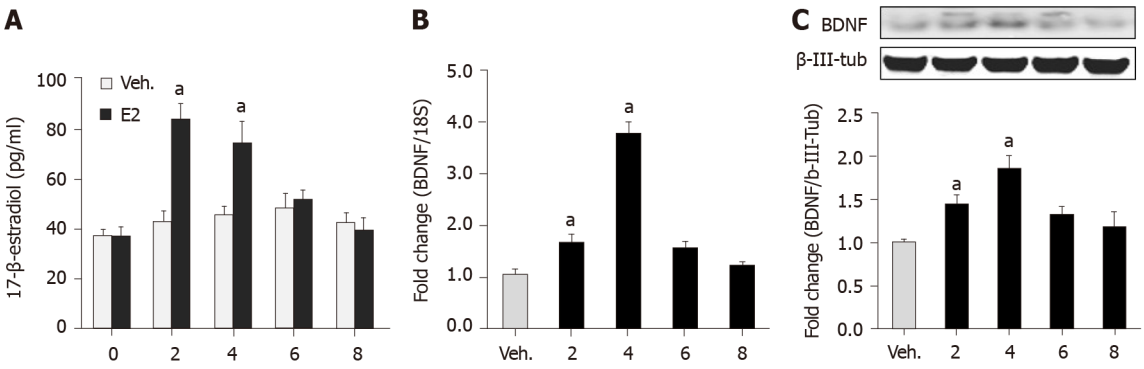


Figure 5 Brain-derived neurotrophic factor expression in lumbar-sacral spinal cord is regulated by estrogen. A: Plasma estrogen levels in cycling females that received a bolus estradiol (E2) infusion on day 1; B: Lumbar-sacral spinal cord brain-derived neurotrophic factor (BDNF) mRNA following bolus estrogen infusion; C: Lumbar-sacral spinal cord BDNF protein following bolus estrogen infusion. (n = 8 rats in each group, two-way ANOVA, $^{a}P < 0.05$ *vs* vehicle group).

significantly reduced visceral hypersensitivity, suggesting that locally produced estrogen in the central nervous system can regulate nociceptive neurons to modulate visceral hypersensitivity. The chronic stress-estrogen-BDNF axis sensitized visceral hypersensitivity in the offspring of females subjected to CPS. The development of chronic stress-induced visceral hypersensitivity in female rats was estrogen dependent. A key component of this hypersensitivity was estrogen-dependent sensitization of primary afferent colon neurons. Our findings provide key scientific evidence in a preclinical model in support of developing gender-based treatment for abdominal pain in IBS.

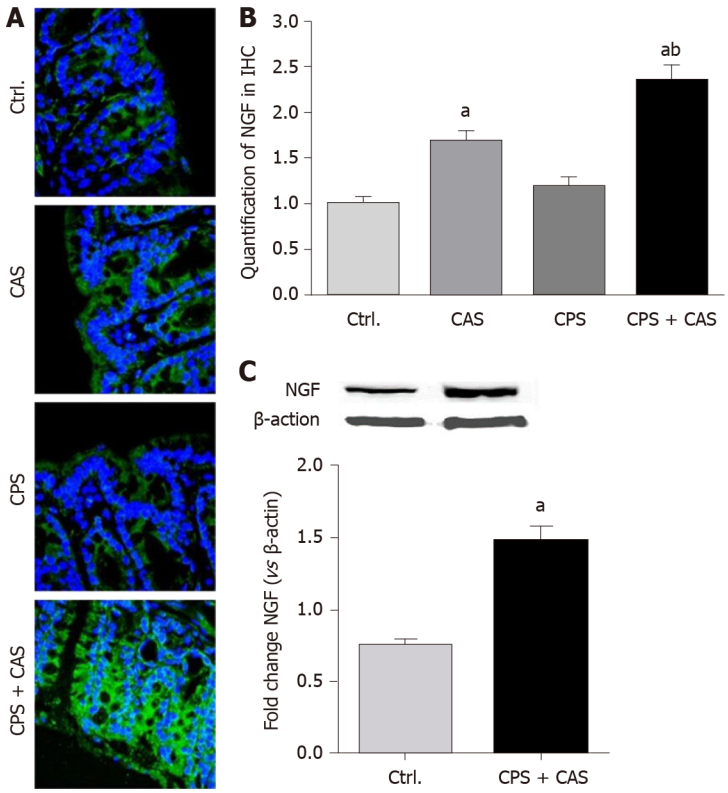


Figure 6 Nerve growth factor expression level in the colon wall. A: Immunohistochemical staining of nerve growth factor (NGF; green) was detected with nuclear counterstaining staining (blue) in controls, chronic adult stress (CAS), chronic prenatal stress (CPS) and CPS + CAS group female rat colon walls. × 400 magnification representative pictures were shown; B: Quantification of NGF levels from colon wall in immunohistochemistry (IHC) (n = 4 rats in each group, one-way ANOVA, $^{a}P < 0.05$ *vs* control group; $^{b}P < 0.05$ *vs* CPS group); C: Western blots of NGF protein from control and CPS + CAS female rats colon wall tissue (n = 6 rats in each group, t-test, $^{a}P < 0.05$ *vs* control group).

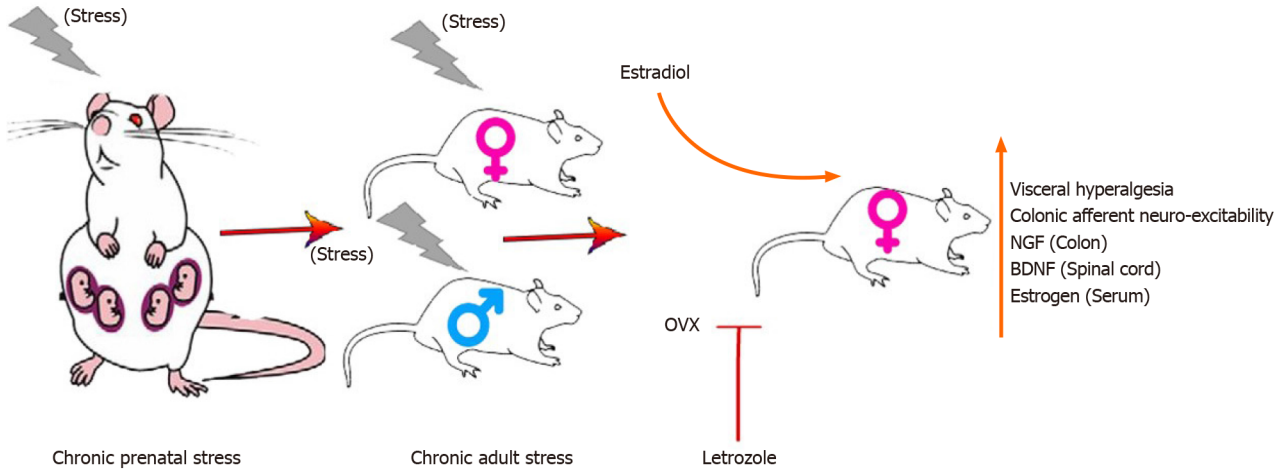


Figure 7 Summary diagram of estrogen re-enhanced visceral hyperalgesia investigated in chronic prenatal stress plus chronic adult stress models. BDNF: Brain-derived neurotrophic factor; NGF: Nerve growth factor.

ARTICLE HIGHLIGHTS

Research background

Chronic stress during pregnancy may increase visceral hyperalgesia in the offspring. Combining adult stress in offspring will increase this sensitivity. Therefore, based on the evidence implicating estrogen exacerbates visceral hypersensitivity in female rodents in preclinical models, we predicted that chronic prenatal stress (CPS) + chronic adult stress (CAS) would maximize visceral hyperalgesia and that estrogen has an important role in colonic hyperalgesia.

Research motivation

The mechanisms of visceral hypersensitivity are not well defined. Understanding the neurophysiological mechanisms driving visceral hypersensitivity will spur the development of female pain-specific therapies.

Research objectives

The objective was to identify the enhancement of visceral hypersensitivity in a CPS + CAS model and explain the role of estrogen in that process.

Research methods

A CPS + CAS rodent model was established. Single fiber recording *in vivo* and patch clamp experiments *in vitro* were used to monitor the activity of colonic neurons. Reverse transcription-polymerase chain reaction (RT-PCR), western blots, and immunofluorescence were used to study the effects of CPS and CAS on colon primary afferent sensitivity. We used ovariectomy (OVX) and letrozole to reduce estrogen levels in female rats in order to assess the role of estrogen in female-specific enhanced primary afferent sensitization.

A CPS + CAS rodent model was established. Single fiber recording *in vivo* and patch clamp experiments were used to monitor the colonic neuron activity in vitro. RT-PCR, western blots, and immunofluorescence were used to study the effects of CPS and CAS on colon primary afferent sensitivity. We used OVX and letrozole to reduce estrogen levels of female rats in order to assess the role of estrogen in female-specific enhanced primary afferent sensitization.

Research results

Spontaneous activity and single fiber activity were significantly greater in females than in males. The enhanced sensitization in female rats mainly came from the low-threshold neurons. CPS significantly increased single-unit afferent fiber activity in the L6-S2 dorsal roots in response. Activity was further enhanced by CAS. In addition, the increased excitability of colon-projecting DRG neurons in CPS + CAS rats was associated with a decrease in transient A-type K+ currents. Compared with OVX, letrozole treatment further reduced the estrogen levels of female rats, which confirmed the gender difference. Moreover, rats treated with letrozole had decreased colonic DRG neuron excitability. The intrathecal infusion of estrogen increased BDNF protein levels and contributed to the response to visceral pain. Western blots showed that nerve growth factor protein was upregulated in CPS + CAS rats.

Research conclusions

This study adds to the evidence of the development of chronic stress-induced visceral hypersensitivity in females, and that it involves estrogen-dependent sensitization of primary afferent colon neurons.

Research perspectives

This study demonstrated that CAS + CPS induced visceral hypersensitivity and that estrogen played a role in the process. Understanding the molecular and neurophysiological mechanisms driving this response will spur the development of female pain-specific therapies.

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ORIGINAL ARTICLE

Clinical and Translational Research

Association of gene and protein expression and genetic polymorphism of CC chemokine ligand 4 in colorectal cancer

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Abstract

BACKGROUND

Leukocytes, such as T cells and macrophages, play an important role in tumorigenesis. CC chemokine ligand (CCL) 4, which is produced by lymphocytes and macrophages, has been found to be expressed in the mucosa of the gastrointestinal tract and is a potent chemoattractant for various leukocytes.

AIM

To examine CCL4 expression and its genetic polymorphism rs10491121 in patients with colorectal cancer (CRC) and evaluate their prognostic significance.

METHODS

Luminex technology was used to determine CCL4 Levels in CRC tissue (n = 98), compared with paired normal tissue, and in plasma from patients with CRC (n = 103), compared with healthy controls (n = 97). Included patients had undergone surgical resection for primary colorectal adenocarcinomas between 1996 and 2019 at the Department of Surgery, Ryhov County Hospital, Jönköping, Sweden. Reverse transcription quantitative PCR was used to investigate the CCL4 gene expression in CRC tissue (n = 101). Paired normal tissue and TaqMan single

nucleotide polymorphism assays were used for the CCL4 rs10491121 polymorphism in 610 CRC patients and 409 healthy controls.

RESULTS

The CCL4 protein and messenger RNA expression levels were higher in CRC tissue than in normal paired tissue (90%, $P < 0.001$ and 45%, $P < 0.05$, respectively). CRC tissue from patients with localized disease had 2.8-fold higher protein expression levels than that from patients with disseminated disease. Low CCL4 protein expression levels in CRC tissue were associated with a 30% lower cancer-specific survival rate in patients ($P < 0.01$). The level of plasma CCL4 was 11% higher in CRC patients than in healthy controls ($P < 0.05$) and was positively correlated ($r = 0.56$, $P < 0.01$) with the CCL4 protein level in CRC tissue. The analysis of CCL4 gene polymorphism rs10491121 showed a difference ($P < 0.05$) between localized disease and disseminated disease in the right colon, with a dominance of allele A in localized disease. Moreover, the rate of the A allele was higher among CRC patients with mucinous cancer than among those with non-mucinous cancer.

CONCLUSION

The present study indicates that the CRC tissue levels of CCL4 and CCL4 gene polymorphism rs10491121, particularly in the right colon, are associated with clinical outcome in CRC patients.

Key Words: CC chemokine ligand 4; Gene polymorphism; Gene and protein expression; Chemokine; Survival rate; Colorectal cancer

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Core Tip: Our data suggest that the CC chemokine ligand (CCL) 4 rs10491121 polymorphism is associated with colorectal cancer (CRC), particularly mucinous CRC and CRC in the right colon. Moreover, we observed that CCL4 protein expression was upregulated in CRC tissues compared with in normal tissues, was correlated with disease stage and that low expression was related to a 30% lower cancer-specific survival rate.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies and one of the leading causes of cancer death[1]. In most cases, the disease occurs sporadically through a transition from dysplastic adenomatous polyps to carcinoma. Genetic alterations and various genetic pathways cause this transition by affecting CRC induction and progression[2,3]. Details of the relationship between inflammation and CRC induction, progression, and metastasis, as well as the importance of tumor suppressor genes in inhibiting carcinogenesis, have been established by us and others [4-6]. Inflammatory factors expressed in CRC cells or in the tumor microenvironment appear to play an important role in local immune regulation and may either promote or suppress CRC pathogenesis. Communication between tumor cells and their microenvironment through secretion of factors is thought to be crucial for tumor growth. Particularly, the interaction between tumor cells and infiltrating leukocytes such as macrophages is vital for CRC progression and prognosis[4,7-9]. There is an ongoing search for molecular biomarkers to facilitate early diagnosis, determine prognosis, and guide the selection of personalized therapy for CRC patients[6,10]. The patients are identified based on pathological and clinical parameters, with CRC

consent was obtained from each of the participants.

Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: The authors declare no conflicts of interest.

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staging primarily based on the tumor-node-metastasis (TNM) system, as described in the American Joint Committee on Cancer classification[11].

The connection between inflammatory factors, such as cytokines and chemokines (chemotactic cytokines), and CRC is well-established[4,12]. Chemokines and their receptors have many roles in immune regulation[13] and some chemokines promote and regulate neoplastic progression, metastasis, angiogenesis, and immune cell infiltration[14-16].

The CC chemokine ligand (CCL) 4, also called macrophage inflammatory protein-1 beta, is a potent chemoattractant for monocytes/macrophages, dendritic cells, and T cells, *via* its cognate receptor CCR5. It is an especially effective chemoattractant for Th1 cells, which have an antitumor role[17-19]. CCL4 is mainly produced by lymphocytes and macrophages[17,18], but is also expressed in the intestinal mucosa and upregulated in gastric cancer and CRC tissue[20-22]. Genetic variations, such as single nucleotide polymorphisms (SNPs), in inflammatory genes are suggested to play a role in CRC risk and the survival of CRC patients[23,24]. The human CCL4 gene is located on chromosome 17 and recent studies have shown a relationship between the nonsynonymous SNP CCL4 rs10491121 (G > A) and oral[25], breast[26], and hepatocellular cancer[27]. To our knowledge, little is known about this SNP and CCL4 expression in patients with CRC. Therefore, the aim of the present study was to examine expression of CCL4 and its genetic polymorphism rs10491121 in patients with CRC and to evaluate their prognostic significance by identifying associations with various clinicopathological parameters and long-term survival.

MATERIALS AND METHODS

Patients and controls

Blood samples were obtained from 610 patients (344 males and 266 females) with a median age of 71 years (range: 25–94 years) who underwent surgical resection for primary colorectal adenocarcinomas between 1996 and 2019 at the Department of Surgery, Ryhov County Hospital, Jönköping, Sweden. Follow-up for the estimation of cancer-specific survival ended on the date of death or on February 23, 2021.

The study included 274 patients with rectal cancer and 336 patients with colon cancer, grouped based on the site of the primary cancer. In accordance with Liang *et al* [28], the colon cancer patients were divided into those with cancer localized in the right colon (cecum, ascending colon, hepatic flexure, transverse colon) (n = 194) and those with cancer localized in the left colon (splenic flexure, descending colon, sigmoid colon) (n = 142). The tumors were classified in accordance with the American Joint Committee on Cancer classification: Stage I in 105 cases, stage II in 228 cases, stage III in 196 cases, and stage IV in 81 cases. The degree of differentiation was categorized as high/medium (478 cases) or poor (132 cases) and the tumors were characterized as non-mucinous (n = 528) or mucinous (n = 82).

Healthy blood donors (n = 347) at Ryhov County Hospital, with no known CRC history and from the same geographical region as the CRC patients, were selected as the control population. This cohort comprised 216 males and 193 females, with a median age of 58 years (range: 33–68). Blood samples were collected at the start of surgery for the patients and at the time of blood donation for the controls. All blood samples were centrifuged to separate plasma and blood cells and then stored frozen, at -70°C, until analysis.

The investigation was approved by the Regional Ethical Review Board in Linköping, Sweden, and informed consent was obtained from each participant.

Genotyping of CCL4 gene polymorphism

DNA was isolated from each blood sample using QiaAmp DNA Blood Kit (Qiagen, Hilden, Germany). The TaqMan SNP genotype assays were used for analysis of the *CCL4* rs10491121 (ID C-11626804-10) genotypes (Applied Biosystems, Foster City, CA, USA). Ten nanograms of DNA were mixed with TaqMan Genotyping Master Mix (Applied Biosystems) and analyzed with the 7500 Fast Real-Time PCR System (Applied Biosystems). Amplification was performed using an initial cycle at 50°C for 2 min, followed by one cycle at 95°C for 10 min, and, lastly, 40 cycles at 95°C for 15 s and at 60°C for 1 min. The manual calling option in the allelic discrimination application ABI PRISM 7500 SDS software version 1.3.1 (Applied Biosystems) was used to assign the genotypes.

Cell lines

Two established human colon cancer cell lines, Caco-2 and HT-29, were purchased from American Type Culture Collection (Rockville, MD, USA). The cell lines were grown in accordance with the supplier's instructions and the growth media were Essential Medium (Caco-2) and Mc Coy 5a (HT-29). The cell lines were stored frozen, at -78°C, until analysis.

Tissue samples and lysates

This study utilized tumor and paired normal tissue samples from 98 of the CRC patients, of whom 52 were males and 46 females, with a median age of 68 years (range: 29–90). The tumors were located in the colon in 53 patients and in the rectum in 45 patients and were classified as stage I in 18 cases, stage II in 33 cases, stage III in 22 cases, and stage IV in 25 cases. Colorectal cancer tissue and adjacent normal colorectal mucosa (within about 5 cm from the tumor) were excised from each patient and immediately frozen at -78°C until analysis. Frozen tumor, paired normal tissue, and cell lines were thawed and homogenized in ice-cold radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, United States) containing a protease inhibitor cocktail, in accordance with the manufacturer's instructions. The lysate was placed on ice for 30 min and then centrifuged at 18000 *g* for 10 min. The protein content of the supernatant fluid was determined for each sample using the Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, United States).

Plasma samples

Of the CRC patients and controls, 103 and 97, respectively, were available for CCL4 protein analysis in plasma. Blood samples were drawn at the start of surgery for the patients and at the time of blood donation for the controls. The CRC patient group comprised 52 males and 51 females with a median age of 68 years (range: 29–90). Forty-six tumors were in the colon and 57 tumors in the rectum; they were classified as stage I in 18 cases, stage II in 34 cases, stage III in 25 cases, and stage IV in 26 cases. Controls for plasma samples included 51 males and 46 females with a median age of 63 years (range: 55–68).

Determination of CCL4 protein levels in tissue, cell lines, and plasma

The levels of CCL4 in plasma, colorectal tissue, and cell line lysates were measured using a Luminex bead-based technology (Bio-Rad Laboratories, Inc.) and commercially available Luminex assay for CCL4 (Bio-Rad Laboratories). The tissue and cell line levels of CCL4 protein were expressed as pg/mg of protein and the plasma CCL4 protein concentration from patients and controls as pg/mL.

Immunohistochemistry

Formalin-fixed and paraffin-embedded CRC tissues from patients with CRC were obtained from the Department of Pathology of the County Hospital Ryhov, Jönköping, Sweden. Among the eight patients, six had tumors localized in the colon and two in the rectum; these were classified as stage I in two cases, stage II in two cases, stage III in two cases, and stage IV in two cases. The staining was performed on 3.5 μm sections of paraffin-embedded tissue samples to detect the CCL4 protein expression. In brief, antigen retrieval was finished by cooking at 110°C for 52 min in Diva Decloaker, 10X (Biocare Medical, Concord, CA, United States). The slides were treated with hydrogen peroxide for 5 min to prevent the occurrence of endogenous peroxidase, which might alter the interpretation of the color. Primary rabbit polyclonal CCL4 antibody against human CCL4 (Abcam, Tokyo, Japan) was used at a dilution of 1:300. The antibody was applied to the tissue sections, which were then incubated for 30 min at room temperature. The MACH 4 Universal HRP-Polymer Detection kit (Biocare Medical) was used, and the reaction was visualized using Betazoid DAB Chromogen Kit (Biocare Medical). Human kidney tissue was used as a positive control for CCL4 expression and rabbit IgG, polyclonal isotype control (Abcam) was used as a negative control and included along with each patient tissue section.

RNA extraction, complementary DNA synthesis, and reverse transcription quantitative PCR

One hundred and one of the CRC patients were available for the analysis of CCL4 messenger RNA (mRNA) expression, of whom 52 were males and 49 females, with a median age of 72 years (range: 43–89). The tumors were located in the colon in 56 patients and in the rectum in 45 patients and were classified as stage I in 16 cases, stage

II in 43 cases, stage III in 38 cases, and stage IV in four cases. Eighty-two available paired normal tissue samples were analyzed.

All CRC tissue samples and paired normal tissue samples were stored in RNA protect tissue reagent (Qiagen) to maintain good RNA quality. RNA was purified using the RNeasy Mini kit (Qiagen), in accordance with the manufacturer's instructions, and eluted in nuclease-free water. The concentration and purity of the RNA and RNA integrity were determined using an Agilent 2100 Bioanalyzer with the Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, United States), in accordance with the manufacturer's instructions. RNA (297 ng) was reverse transcribed in a total volume of 20 μL using Super Script III Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific, Carlsbad, CA, United States), in accordance with the manufacturer's instructions. The resulting complementary DNA and the remaining RNA were stored at -80°C.

Complementary DNA was amplified through reverse transcription quantitative PCR with TaqMan™ Fast Universal PCR Master Mix (Cat.no 4366072, Applied Biosystems) and TaqMan Gene Expression Probes and Primers (RPLP0 Cat.no HS99999902 m1 and CCL4 Cat.no HS99999148 m1, Applied Biosystems) on a QuantStudio5 Real Time PCR system (Applied Biosystems), with each sample run in duplicate. Results were normalized to expression levels of human RPLP0. Relative quantification of gene expression was performed using the standard curve method and expressed in arbitrary units.

Statistical analysis

The differences in the frequencies of the CCL4 gene polymorphism between CRC patients and controls and between clinical characteristics within the CRC subgroups were analyzed using the chi-squared test. The Hardy-Weinberg equilibrium was assessed for the genotypes. Survival analysis was performed through Kaplan-Meier analysis with log-rank test. The Wilcoxon's signed-rank test and the Mann-Whitney *U*-test were used for the analysis of the related and independent parameters and the Kruskal-Wallis test was used for comparing three or more classes. Correlations between parameters were analyzed using Spearman's rank correlation test. Statistical analyses were performed using Stata Statistical Software Release 15 (Stata Corp., College Station, TX, United States) and SPSS software for Windows, version 14.0 (SPSS Inc., Chicago, IL, United States). Results were considered statistically significant at $P < 0.05$. The statistical methods of this study were reviewed by Roland E Andersson from Region Jönköping County, Sweden.

RESULTS

CRC risk, clinicopathological features, and survival in relation to CCL4 gene polymorphism

No statistically significant differences in the genotypic or allelic frequencies were observed between the patients and the healthy control group for CCL4 rs10491121 (Table 1). Stratification analysis of associations between this SNP and the tumor location showed no statistically significant differences between colon and rectum, or between right and left colon (data not shown). However, we noted a difference between localized (stages I + II) and disseminated (stages III + IV) cancer in the right colon, but not in the left colon, regarding the genotypic ($P = 0.003$) and allelic ($P = 0.013$) distributions (Table 2). The presence of allele A in the right colon was more common in stages I + II (40.5%) than in stages III + IV (28.2%) (Table 2) with an odds ratio (OR) = 1.73 (95% confidence interval = 1.12–2.68, $P = 0.013$). Analysis of the association between the genotypic or allelic distributions in non-mucinous or mucinous CRC showed statistically significant differences (Table 3). The rate of the A allele was higher (45.1%) among patients with mucinous cancer than among those with non-mucinous cancer (34.6%) with an OR = 1.56 (95% confidence interval = 1.12–2.17, $P = 0.009$).

Based on data from our cohort with up to 25 years follow-up, the Kaplan-Meier analysis revealed no differences in cancer-specific survival overall. No significant association was found between the genotypes or alleles when patients were stratified by gender, age, or degree of differentiation (data not shown). Neither the patient group nor the control group showed significant deviation from the Hardy-Weinberg equilibrium (data not shown).

Table 1 Genotypic and allelic distributions of the CC chemokine ligand 4 gene polymorphism (rs10491121) in *n* (%), among 610 patients with colorectal cancer and 409 healthy controls

Genotype/allele	Patients	Controls
G/G	(250 (41.0)	176 (43.0)
G/A	281 (46.1)	181 (44.3)
A/A	79 (12.9)	52 (12.7)
G	781 (64.0)	533 (65.2)
A	439 (36.0)	285 (34.8)

CCL4: CC chemokine ligand 4.

Table 2 Genotypic and allelic distributions of the CC chemokine ligand 4 gene polymorphism (rs10491121) in *n* (%), regarding tumour location and disease stage among patients with colon cancer

Variable	Cases	Genotype			Allele	
Right colon		G/G	G/A	A/A	G	A
Stages I + II	116	38 (32.8)	62 (53.4)	16 (13.8)	138 (59.5)	94 (40.5)
Stages III + IV	78	44 (56.4)[b]	24 (30.8)	10 (12.8)	112 (71.8)[a]	44 (28.2)
Left colon						
Stages I + II	67	23 (34.3)	34 (50.8)	10 (14.9)	80 (59.7)	54 (40.3)
Stages III + IV	75	27 (36.0)	37 (49.3)	11 (14.7)	91 (60.7)	59 (39.3)

[a]$P < 0.05$.
[b]$P < 0.01$. CCL4: CC chemokine ligand 4.

Table 3 Genotypic and allelic distributions of the CCL4 gene polymorphism (rs10491121) in *n* (%), regarding non-mucinous and mucinous cancer among patients (*n* = 610) with colorectal cancer

Genotype/allele	Non-mucinous	Mucinous
G/G	222 (42.0)	28 (34.1)
G/A	247 (46.8)	34 (41.5)
A/A	59 (11.2)	20 (24.4)[b]
G	691 (65.4)	90 (54.9)
A	365 (34.6)	74 (45.1)[b]

[b]$P < 0.01$.
CCL4: CC chemokine ligand 4.

Protein expression in CRC tissue and plasma in relation to clinical features and survival

The level of CCL4 protein was 90% higher in CRC tissue compared with in normal paired tissue ($P < 0.001$; Table 4). Moreover, the level of plasma CCL4 was 11% higher in patients than in controls ($P < 0.050$; Table 4) and showed a positive correlation ($r = 0.56$, $P < 0.01$) with levels in the CRC tissue.

The CRC tissue showed a gradual decrease in CCL4 protein level in relation to disease stage, and localized disease (stages I + II) showed a 2.8-fold higher CCL4 protein level than disseminated disease (stages III + IV, $P = 0.001$; Table 5). There was no difference in tissue levels of CCL4 protein for any of the other reported clinical features such as age, gender, location, differentiation grade, or mucinous state. Moreover, plasma levels of CCL4 were not associated with any of the investigated clinical features (data not shown). In the colon cancer cell lines, we noted that the

Table 4 Tissue levels and plasma levels of CC chemokine ligand 4 in colorectal cancer patients

Variable	Cases, *n*	CCL4 protein
Tissue		
Colorectal cancer tissue	98	137 [5–3413] pg/mg[b]
Paired normal tissue	98	72 [5–2205] pg/mg
Plasma		
Patient	103	63 [8–3428] pg/mL[a]
Control	97	57 [2–178] pg/mL

Data are shown as median [range].
[a]$P < 0.05$.
[b]$P < 0.001$. CCL4: CC chemokine ligand 4.

Table 5 Disease stage in relation to cancer tissue levels of CC chemokine ligand 4 in colorectal cancer patients

Variable	Cases, *n*	CCL4 protein in pg/mg
Stage		
I	18	262 [12–3326]
II	33	188 [5–2797]
III	22	125 [24–3413]
IV	25	71 [10–1188][b]
I + II	51	235 [5–3326]
III + IV	47	84 [10–3413][b]

Data are shown as median [range].
[b]$P < 0.001$. CCL4: CC chemokine ligand 4.

average protein level of CCL4 was 7.8 pg/mg in HT-29 and 2.9 pg/mg in Caco-2.

When CRC tissue levels of the CCL4 protein were used to group patients into tertiles (with low and medium/high levels), we found that the patients with low tissue levels of the CCL4 had a 30% lower cancer-specific survival rate ($P = 0.005$, log-rank test, Figure 1).

CCL4 is expressed in epithelial cells

Immunohistochemical evaluation of CCL4 expression in CRC and normal tissue showed positive and negative to weak staining, respectively (Figure 2A-D). Cytoplasmic staining was evident predominantly in the epithelial cells. The staining of slides with isotype antibody control and with secondary antibody revealed only negative staining (Figure 2E and F).

CCL4 mRNA expression in CRC and paired normal tissue

The CCL4 mRNA expression was 45% higher in CRC tissue (median 61 AU, range: 2–494) compared with normal paired tissue (median 42 AU, range: 4-160, $P = 0.044$). When the relationship between the CCL4 mRNA expression and the mucinous state of the CRC tissue was analyzed, the expression level was found to be higher in mucinous CRC tissue (median 78 AU, range: 11–243) than in non-mucinous CRC tissue (median 56 AU, range: 2–494, $P = 0.043$). CCL4 mRNA expression levels were not associated with other reported clinical features (data not shown).

DISCUSSION

Previous studies have shown that CCL4 gene polymorphism rs10491121 (G > A) is associated with susceptibility to several cancer diseases[25-27], but little is known

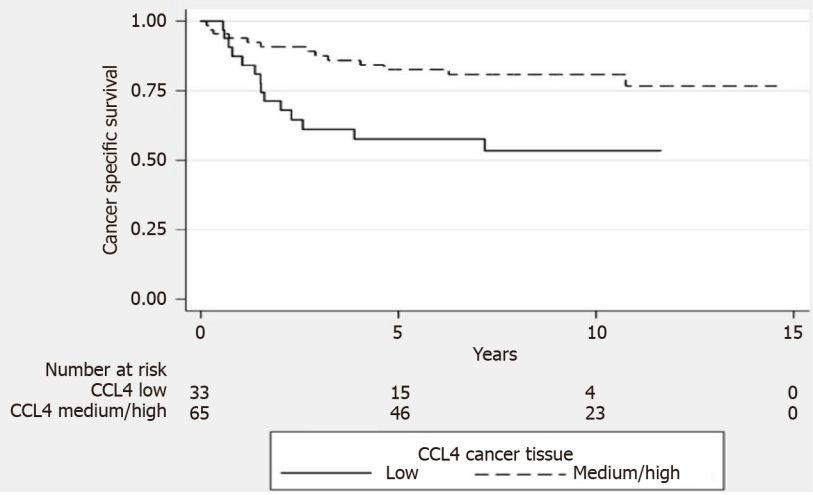


Figure 1 Kaplan-Meier curves between expression levels of CC chemokine ligand 4 protein grouped into tertiles (low and medium/high) and cancer-specific survival rates in colorectal cancer patients. CCL4: CC chemokine ligand 4.

about this SNP in relation to CRC. The present study found no statistically significant differences between CRC patients and controls in genotype distribution or allelic frequencies of this SNP. Further, no associations were identified with clinical characteristics other than location, stage, and mucinous state. The genotypes of CCL4 gene polymorphism rs10491121 may influence gene expression, protein levels, and protein function of CCL4. However, to the best of our knowledge, there is limited data to describe the functional activity of this SNP. Regarding cancer, it has previously been shown that patients carrying the AG or GG genotype are at lower risk of developing distant metastases in breast cancer[26]. In our study, stratification analysis showed a difference in the frequency of this SNP between localized disease and disseminated disease in the right colon, but not in the left colon. Specifically, in the right colon, a dominance of the allele A was seen in localized disease as compared with in disseminated disease. Rectal, left, and right colon cancer differ with respect to histology, clinical outcomes, and expression of distinguishable genes, which has led to the hypothesis that they constitute different disease entities[28-30]. In our study, it is possible that the genotype distribution of CCL4 gene polymorphism rs10491121, together with other gene or gene-environment interactions, contributed to development towards localized or disseminated disease in the right colon.

Mucinous cancer is a subtype of CRC and is more common in proximal colon cancer than in distal colon and rectal cancer. There are conflicting results in the literature regarding the prognosis and survival rates for mucinous CRC[31]. Factors involved in the development of mucinous CRC are not yet known, but mucinous cancer has a different molecular signature than non-mucinous cancer[31]. This study demonstrated that the *CCL4* gene polymorphism rs10491121 was associated with mucinous CRC, with a higher rate of the A allele than non-mucinous cancer. Moreover, we noted that higher CCL4 mRNA expression levels were associated with mucinous cancer. As far as we know, this is the first time data relating to this investigated genetic variant of CCL4 and CCL4 gene expression in mucinous CRC have been published. Further studies are needed to verify our findings and to investigate the potential function of this SNP on CCL4 expression and for prediction of the mucinous state.

Higher CCL4 mRNA expression levels in tumor tissue than in normal tissue in colon (P = 0.07) was suggested in a previous study including 20 patients[21]. In the present study, with 101 CRC patients, we confirmed that the CCL4 mRNA expression levels were higher (P = 0.044) in CRC tissue than in normal tissue. Two studies focused on CCL4 protein expression found higher levels in CRC tissue compared with in adjacent normal tissue[20,32]. These studies included only 10[20] and 25 patients[32], respectively, and found no association between CCL4 protein levels and cancer stage. In our study, CCL4 protein expression levels were examined in a large series of 98 patients with CRC. We found that CCL4 protein levels were higher in CRC tissue than in normal paired tissue. We also found that localized disease was associated with higher CCL4 protein levels than disseminated disease. The interaction between tumor cells and infiltrating leukocytes such as macrophages is a powerful relationship that affects CRC progression and prognosis[4,7-9]. CCL4 protein can be produced by lymphocytes and macrophages[17,18] and, in this study, immunostaining revealed

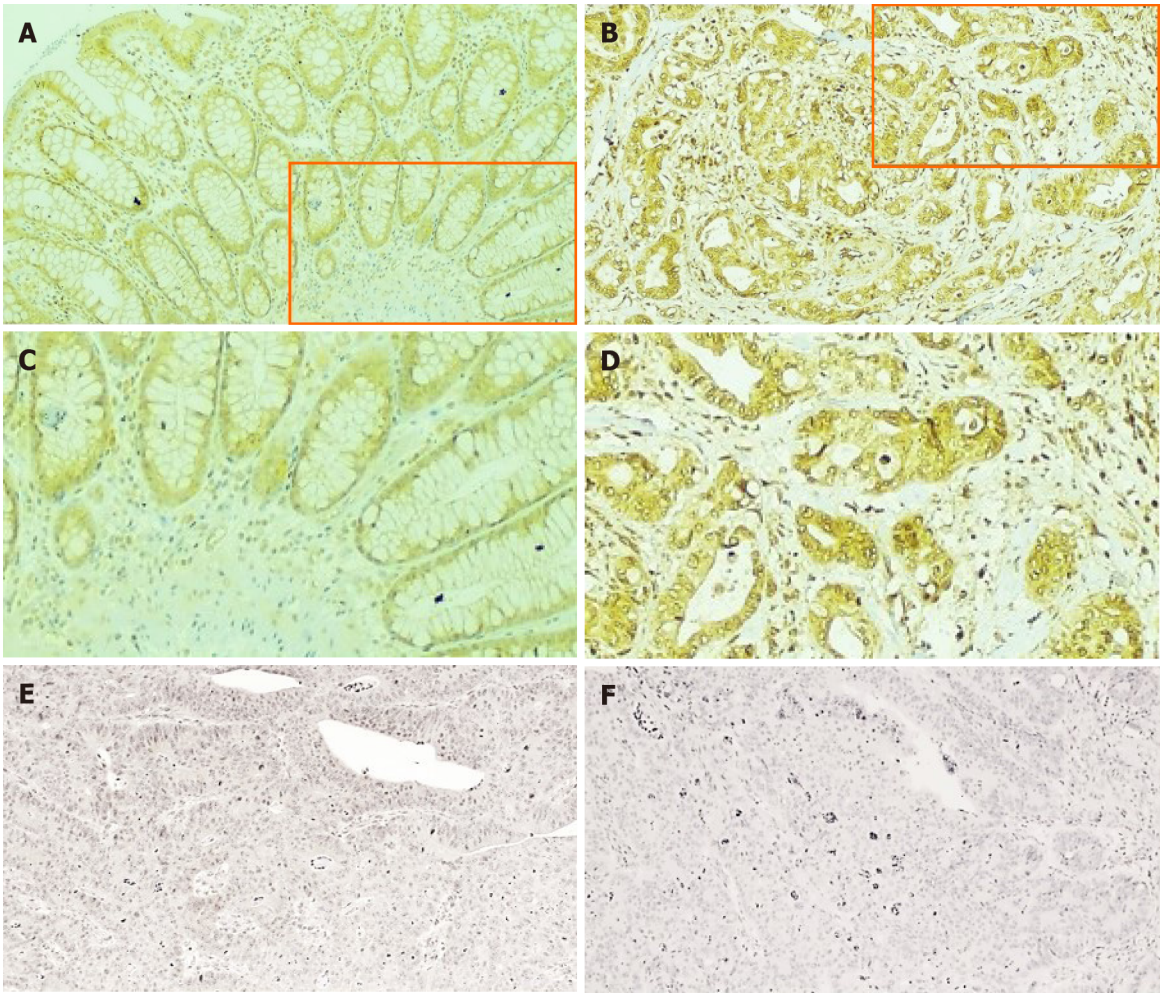


Figure 2 Representative immunohistochemical staining of CC chemokine ligand 4. A: Normal tissue (× 100 magnification); B: Colon cancer tissue (× 100 magnification); C: Normal tissue (× 400 magnification) of highlighted area; D: Colon cancer tissue (× 400 magnification) of highlighted area; E: Isotype control antibody in colon cancer tissue (× 100 magnification); F: Primary antibody omitted in colon cancer tissue (× 100 magnification).

expression of CCL4 in CRC tissue predominantly in epithelial cells. We also detected CCL4 protein in the colon cancer cell lines HT-29 and Caco-2. The lack of association between CCL4 mRNA and CCL4 protein levels in the present study could be explained by differences in transcription rate, mRNA stability, or translation efficiency within cell types such as lymphocytes, macrophages, and epithelial cancer cells in the colon and rectum.

When we grouped CRC tissue levels of the CCL4 protein as low or medium/high, we found that patients with low CCL4 protein levels in CRC tissue had lower cancer-specific survival rates. Thus, based on our data, higher CCL4 protein levels in CRC tissue are associated with less advanced disease stage and better prognosis. The properties of the tumor microenvironment and the interactions between tumor cells and infiltrating leukocytes, such as macrophages and T cells, affect CRC progression and prognosis[4,7-9]. Tumor-associated macrophages (TAMs) are commonly found in CRC tissue[7,8]. Macrophages are an important component of the tumor microenvironment and coordinate various aspects of immunity. TAMs promote tumor growth by facilitating angiogenesis, immunosuppression, and inflammation, and can also influence tumor relapse after anticancer therapies. Depending on activation status, macrophages can exert dual influences on tumorigenesis, either by antagonizing the cytotoxic activity in immune cells or by enhancing antitumor responses[7,8]. The classic view is that TAMs have one of two phenotypes, M1 or M2, which have antitumoral or protumoral activities, respectively[7,8].

The chemokine CCL4 is a potent chemoattractant for monocytes/macrophages, dendritic cells, and T cells, *via* its cognate receptor CCR5, and it is an especially effective chemoattractant for Th1 cells, which have an antitumor role[17-19]. TAMs adapt their phenotype in response to environmental stimuli and the presence of interferon gamma and tumor necrosis factor alpha (TNF-α) promotes polarization into

phenotype M1[7,8]. In a previous study, we showed that TNF-α protein level was significantly higher in CRC tissue than in normal tissue[5]. It could be speculated that a higher level of CCL4 increases the prevalence of Th1 and TAMs, with a shift towards M1 macrophages that is stronger at lower stages than at higher stages, thus negatively affecting tumor development. However, the impact of macrophages in tumor progression remains to be fully elucidated, in part due to the contrasting roles they play depending on their polarization[7,8]. Further studies with immunohistochemical approaches will be conducted to clarify the number of TAMs, their phenotypic patterns, and the presence of T lymphocytes in our analyzed CRC tissue. From a molecular standpoint, the underlying mechanisms responsible for the differing levels of CCL4 at different disease stages remain to be clarified.

This study demonstrated that levels of plasma CCL4 were higher in patients compared with controls and were positively correlated with levels in CRC tissue. Plasma CCL4 concentration could possibly reflect levels in CRC tissues. However, we failed to detect a stage-dependent alteration of plasma CCL4 Level, which is consistent with data from another study[33]. Due to the general immunological imbalance indicated by the systemic cytokine pattern in CRC patients[33,34], we hypothesize that the production of CCL4 by lymphocytes and monocytes may be altered. Thus, the relationship between concentration of CCL4 in plasma and cancer stage may be masked.

Some limitations of our study should be mentioned. It is important to note that this study was of an exploratory nature. The patients and controls were selected from one hospital and may not represent the general populations. Additional studies with larger and more diverse populations of patients and controls are needed to validate our findings.

CONCLUSION

Our data suggested that the CCL4 rs10491121 polymorphism was associated with CRC, particularly mucinous CRC and CRC in the colon. Moreover, we observed that CCL4 protein expression was upregulated in CRC tissues compared with in normal tissue, correlated with disease stage, and that low expression was related to a lower cancer-specific survival rate. Detailed functional analysis is required to reveal the mechanisms underlying our observed associations that different levels of CCL4 in CRC tissue reflect different stages of disease. Moreover, studies with immunohistochemical approaches will be conducted to clarify the contribution of TAMs and their phenotypic patterns and the presence of T lymphocytes in our cohort of CRC tissue.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer (CRC) is one of the most common malignancies and one of the leading causes of cancer death. Inflammatory factors expressed in CRC cells or in the tumor microenvironment play an important role in local immune regulation. Among these factors, chemokines like CC chemokine ligand (CCL) 4 play an important role in facilitating recruitment of leukocytes.

Research motivation

There is an ongoing search for molecular biomarkers to facilitate early diagnosis, and to determine the prognosis of CRC patients.

Research objectives

The aim of the present study was to examine expression of CCL4 and its genetic polymorphism rs10491121 in patients with CRC and to evaluate their association to clinicopathological parameters and prognostic impact.

Research methods

Blood, tumor and paired normal tissue from patients with CRC and blood samples from healthy controls were subjected to an analysis of CCL4 protein using Luminex technology. Reverse transcription quantitative PCR was used to investigate the CCL4 gene expression in CRC tissue and paired normal tissue. For studies on the CCL4

rs10491121 polymorphism in CRC patients and healthy controls, TaqMan genotype assays based on polymerase chain reaction were used.

Research results

The CCL4 protein and messenger RNA expression levels were higher in CRC tissue than in normal paired tissue and the level of CCL4 protein in CRC tissue from patients with localized disease was higher than that in CRC tissue from patients with disseminated disease. Low levels of CCL4 protein in CRC tissue were associated with a 30% lower cancer-specific survival rate in patients. The level of CCL4 in plasma was higher in CRC patients than in healthy controls and was positively correlated with the CCL4 protein level in CRC tissue. There was a difference in polymorphism rs10491121 between localized disease and disseminated disease in the right colon, with a dominance of allele A in localized disease.

Research conclusions

The results from this study indicate that CCL4 expression and gene polymorphism are good markers of prognosis. However, the findings need to be reproduced in larger cohorts.

Research perspectives

The chemokine CCL4 deserves further attention as a clinical prognostic biomarker in CRC. Detailed functional analysis is required to reveal the mechanisms underlying the observed associations between different levels of CCL4 in CRC tissue and different stages of disease.

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ORIGINAL ARTICLE

Retrospective Study

Clinical efficacy of antiviral therapy in patients with hepatitis B-related cirrhosis after transjugular intrahepatic portosystemic shunt

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Author contributions: Yao X performed the operation and wrote the paper; Zhou H performed the operation and collected the data; Tang SH collected and analyzed the data; Huang S performed the operation and analyzed the data; Qin JP participated in and guided the operation and finalized the manuscript.

Institutional review board statement: This study was approved by the Hospital Ethics Committee.

Informed consent statement: Patients and their families were fully informed and provided signed consent for surgery.

Conflict-of-interest statement: The authors declare no competing interests for this manuscript.

Data sharing statement: The study protocol, statistical analysis plan, and de-identified participant data can be made available upon request for non-commercial purposes and after approval of a study proposal through a signed

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Abstract

BACKGROUND

As a country with a high burden of hepatitis B, China has about 86 million cases of hepatitis B virus infection, ranking the first in the world. Currently, there are about 390000 deaths due to hepatitis B-related complications such as liver cirrhosis and liver cancer every year. Consequently, how to control portal hypertension, improve liver functional reserve, and reduce the incidence of hepatic failure and liver cancer in such patients is the focus of current clinical attention. Previous clinical study in our center suggested that at 24 mo after transjugular intrahepatic portosystemic shunt (TIPS), the liver functional reserve of patients with hepatitis B cirrhosis was better than that of patients with alcohol-induced and immune cirrhosis, which may be related to the effective etiological treatment.

AIM

To investigate the clinical efficacy of three first-line antiviral drugs recommended by the guidelines of prevention and treatment for chronic hepatitis B in China (2019) in the treatment of patients with hepatitis B-related cirrhosis who had received a TIPS.

METHODS

The clinical data of 137 patients with hepatitis B-related cirrhosis with portal hypertension after receiving TIPS at our centre between March 2016 and December 2020 were analysed retrospectively. According to different anti-viral drugs, the patients were divided into entecavir (ETV) (n = 70), tenofovir alafenamide fumarate (TAF) (n = 32), and tenofovir disoproxil fumarate (TDF) (n = 35) groups. The cumulative incidence of hepatic encephalopathy and hepatocellular carcinoma, survival, and changes in hepatic reserve function and

glomerular filtration rate in patients treated with different antiviral drugs within 24 mo after surgery were investigated.

RESULTS

At 24 mo after surgery, the Child–Pugh score in the TAF group (6.97 ± 0.86) was lower than that in the TDF (7.49 ± 0.82; t = -2.52, P = 0.014) and ETV groups (7.64 ± 1.17; t = -2.92, P = 0.004). The model for end-stage liver disease score in the TAF group at 24 mo after surgery was 9.72 ± 1.5, which was lower than that in the TDF (10.74 ± 2.33; t = -2.09, P = 0.040) and ETV groups (10.97 ± 2.17; t = -2.93, P = 0.004). At 24 mo after surgery, the estimated glomerular filtration rate (eGFR) in the TAF group (104.41 ± 12.54) was higher than that in the TDF (93.54 ± 8.97) and ETV groups (89.96 ± 9.86) (F = 21.57, P < 0.001).

CONCLUSION

At 24 mo after surgery, compared with TDF and ETV, TAF has significant advantages in the improvement of liver functional reserve and eGFR.

Key Words: Transjugular intrahepatic portosystemic shunt; Hypertension; Antiviral; Hepatitis B-related cirrhosis; Liver reserve function

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Core Tip: As a country with a high burden of hepatitis B, China has about 86 million cases of hepatitis B virus (HBV) infection, ranking the first in the world. Hepatitis B cirrhosis complicated with portal hypertension is characterized by persistent HBV replication and aggravated liver inflammation and fibrosis. Considering the fact that there is currently no report on the clinical efficacy of antiviral therapy for patients with hepatitis B cirrhosis after transjugular intrahepatic portosystemic shunt, we believe that this study has appreciated clinical reference value for the selection of anti-HBV drugs in such patients.

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INTRODUCTION

Patients with chronic hepatitis B (CHB) cirrhosis complicated with severe portal hypertension-related complications rarely accept liver transplantation because of limited liver source and high medical costs. As a result, how to improve the liver functional reserve, reduce the incidence of liver cancer, and enhance the survival rate of such patients has become the focus of clinical research. Transjugular intrahepatic portosystemic shunt (TIPS) is an effective method to treat the complications secondary to liver cirrhosis complicated with portal hypertension. While portal hypertension-related complications in patients with CHB cirrhosis are effectively controlled, early initiation of antiviral therapy can significantly reduce long-term liver-related complications, such as hepatocellular carcinoma (HCC) and hepatic failure, and improve the survival rate. At present, tenofovir disoproxil fumarate (TDF), entecavir (ETV), and tenofovir alafenamide fumarate (TAF) are recommended as the first-line treatment options for CHB patients by major international and domestic guidelines for the diagnosis, treatment, and management of CHB. Moreover, previous clinical study[1] in our center suggested that the use of an 8-mm stent during TIPS could not only effectively shunt, but also relieve portal pressure. Twenty-four months after surgery, the liver functional reserve of patients with hepatitis B cirrhosis was better than that of patients with alcohol-induced and immune cirrhosis, which may be related to the effective etiological treatment. Based on the above studies, the clinical data of patients with hepatitis B cirrhosis after TIPS treated with different antiviral drugs were

data access agreement. Proposals should be directed to jpqqing@163.com.

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retrospectively analyzed in this study. However, TAF entered the Chinese market since November 2018. Current data on the efficacy and safety of TAF in Chinese people are rare, especially in patients with CHB cirrhosis complicated with portal hypertension whose portal vein pressure is relieved after TIPS. Therefore, we evaluated the clinical efficacy of the three antiviral drugs through this retrospective study, with an aim to provide reference for antiviral treatment of patients with CHB cirrhosis after TIPS.

MATERIALS AND METHODS

Subjects

The clinical data of 137 patients with CHB cirrhosis complicated with portal hypertension treated by TIPS with a Viator stent at our center from March 2016 to December 2020 were collected. The inclusion criteria included: (1) Adult patients with CHB cirrhosis complicated with portal hypertension treated by TIPS for the first time; (2) patients receiving TIPS with a shunt channel dilated by an 8-mm balloon and an 8-mm TIPS-specific stent (Viator stent) implanted; and (3) patients undergoing monotherapy (ETV/TDF/TAF) for hepatitis B virus (HBV) for more than one year, with complete virological response maintained 3, 6, and 12 mo after surgery, as evidenced by a low HBV DNA (the lower limit of detection of HBV DNA was 100 IU/mL). The exclusion criteria were as follows: (1) Patients with liver cancer confirmed by imaging examination before surgery, and a history of splenectomy or partial splenic artery embolization; (2) patients with stent dysfunction; (3) patients with preoperative Child-Pugh score for hepatic function > 13 and underlying nephropathy [estimated glomerular filtration rate (eGFR) < 90 mL/min]; (4) patients with cavernous transformation of the portal vein or portal thrombosis; (5) patients with preoperative hepatic encephalopathy (HE), hepatorenal syndrome, and hepatopulmonary syndrome; (6) patients with a history of major liver surgery such as hepatectomy, surgical shunt, and liver transplantation; (7) patients with severe coagulation disorders; (8) patients with severe right heart failure and pulmonary hypertension; (9) patients with uncontrolled intrahepatic infection [alanine aminotransferase (ALT) was more than 10 times higher than the upper limit of normal] and systemic infection; (10) pregnant and lactating patients or patients with pregnancy plan; and (11) patients with liver cirrhosis treated with warfarin and other anticoagulants after surgery. All the patients met the indications of the United States TIPS guidelines[2] and the American Association of the Study of Liver Diseases (AASLD) guidelines for the prevention and treatment of CHB[3]. This study was approved by the hospital ethics committee. The patients and their families were fully informed and signed an informed consent form.

Equipment and materials

Digital subtraction angiography (DSA) was carried out using the AXIOM-Artist DSA system (Siemens, Germany) and Mark V high-pressure syringes. Surgical materials included a RUPS-100 puncture kit, a straight-needle multi-side hole catheter, an Opta Pro balloon catheter, embolization coils, a Cobra catheter (Cook, United States), a stiffening exchange guide wire, and a Viatorr stent (Gore and Associates, United States). Additionally, TDF (300 mg/tablet, Beite Pharmaceutical Co., Ltd.), ETV tablets (0.5 mg/tablet, Sino-American Shanghai Squibb Pharmaceutical Co., Ltd.), and TAF tablets (25 mg/tablet, Gilead, United States) were used in this study.

Research methodology

Preoperative preparation: Preoperatively, routine examinations including routine blood test, liver and kidney function test, HBV-DNA quantitation, and prothrombin time test were performed. All the patients underwent enhanced computed tomography (CT) of the liver and three-dimensional (3D) reconstruction of the hepatic vein-portal vein. The anatomic relationship between the hepatic vein and the portal vein was analyzed to guide the puncture of the portal vein during surgery.

TIPS approach: All the patients received TIPS *via* the right jugular vein. Under the guidance of a guide wire, the puncture system passed through the superior vena cava and the right atrium to the right hepatic vein or the hepatic segment of the inferior vena cava. According to the images obtained from preoperative enhanced CT of the liver and 3D reconstruction of the hepatic vein-portal vein, the puncture of the portal vein branch was guided. After the safety of the puncture was evaluated, portal vein

pressure was measured and the collateral circulation vessels causing esophagogastric varices were embolized. Then, a stent was inserted after balloon dilation puncturing the channel, followed by another portal vein angiography and measurement of portal vein pressure. The detailed operations have been reported in a previous study[4]. All operations were successfully completed by the same group of professionals using an 8-mm balloon and an 8-mm Viatorr stent, without severe operation-related complications.

Postoperative follow-up: The data on HBV-DNA quantitation, liver and kidney function, and coagulation were collected before surgery and 1, 3, 6, 12, and 24 mo after surgery. Ascites and HE were understood by ultrasonography and follow-up records. Child-Pugh grade and the model for end-stage liver disease (MELD) were obtained, respectively. The follow-up endpoints of follow-up was death at 24 mo after surgery, no response to follow-up for consecutive 2 times or more, liver transplantation, or study deadline (December, 2020). The evaluation methods of follow-up were referred to the references[5-7].

Statistical analysis

All measurement data are expressed as the mean ± SD or percentages. The portal pressure gradient before and after treatment was analyzed using the paired *t*-test or chi-square test. The incidence of HE and liver cancer and survival rate were calculated with the Kaplan-Meier curve and compared by the log-rank test. All the data were statistically analyzed using SPSS 22.0. $P < 0.05$ was considered significantly significant.

RESULTS

Patient characteristics

A total of 137 patients with liver cirrhosis were enrolled, including 75 males and 62 females, with an average age of 54.0 ± 9.1 years. According to different antiviral drugs, the patients were divided into an ETV group ($n = 70$), a TAF group ($n = 32$), and a TDF group ($n = 35$). Gender, age, preoperative and postoperative portal pressure gradient, preoperative and postoperative Child-Pugh grade, MELD score, the proportion of hepatitis B e antigen (HBeAg)-positive patients, median HBV DNA level, median time to TAF/TDF/ETV therapy, and kidney function index (eGFR or sCr) showed no statistically significant differences among the three groups (Table 1).

TIPS operations

TIPS *via* the right jugular vein was performed successfully in all the 137 patients, without severe intraoperative complications such as abdominal hemorrhage and bile duct hemorrhage. The collateral circulation causing esophagogastric varices was embolized and the stent was implanted successfully. The success rate of surgery was 100%, and the short-term hemostasis rate was 100%. The portal pressure gradient (mmHg) decreased from 25.71 ± 5.32 preoperatively to 9.95 ± 2.75 postoperatively ($t = 37.32$, $P < 0.001$).

Survival, HE, and liver cancer in three groups

The median follow-up time was 24 (20, 24) mo. The survival rates at 12 and 24 mo after surgery was 93.3% and 89.6% in the TAF group, 93.9% and 86.9% in the TDF group, and 91.2% and 84.3% in the ETV group, respectively (log rank $\chi^2 = 0.517$, $P = 0.772$) (Figure 1A). During the follow-up, a total of 17 patients died, with a median time to death of 12 (7, 20) mo, including three cases in the TAF group, four in the TDF group, and ten in the ETV group. The causes of death included primary liver cancer in 11 (2/2/7) patients, sepsis/infection in 4 (1/1/2), and hepatorenal syndrome in 2 (0/1/1).

One, three, six, twelve, and twenty-four months after surgery, the incidence of HE was 6.2%, 12.5%, 12.5%, 15.6%, and 19.5% in the TAF group, 2.9%, 14.3%, 14.3%, 17.6%, and 25.7% in the TDF group, and 5.7%, 13.1%, 16.1%, 20.9%, and 28.3% in the ETV group (log rank $\chi^2 = 0.712$, $P = 0.700$) (Figure 1B). During the follow-up, HE occurred in 32 patients, with a median time to occurrence of 3 mo. There were six cases of HE in the TAF group (HE grade I/II/III-IV, 4/2/0), eight in the TDF group (HE grade I/II/III-IV, 6/1/1), and 18 in the ETV group (HE grade I/II/III-IV, 14/2/2).

Table 1 Basic clinical data

Clinical characteristic	TAF (*n* = 32)	TDF (*n* = 35)	ETV (*n* = 70)	Statistic	*P* value	Total (*n* = 137)
Gender, *n* (%)				$X^2 = 1.033$	0.597	
Male	20	18	37			75
Female	12	17	33			52
Age (yr, mean ± SD)	53.8 ± 8.5	54.4 ± 7.3	54.5 ± 10.3	$t = 0.234$	0.815	54.3 ± 9.2
Child-Pugh classification				$X^2 = 1.623$	0.805	
A	9	14	21			44
B	18	17	37			72
C	5	4	12			21
Child-Pugh score	6.94 ± 1.29	7.46 ± 2.14	7.26 + 1.64	F = 1.119	0.330	7.31 ± 1.75
MELD score	10.37 ± 2.94	9.69 ± 2.96	10.41 ± 2.93	F = 0.775	0.463	10.22 ± 2.93
Prothrombin time (s)	14.40 ± 1.86	15.14 ± 2.81	14.95 ± 2.73	F = 0.930	0.354	14.74 ± 2.59
Albumin (g/L)	35.27 ± 5.65	34.14 ± 5.01	34.43 ± 4.33	F = 0.496	0.610	34.55 + 4.82
eGFR (mL/min/1.73 m^2)	91.22 ± 10.67	90.37 ± 14.24	90.19 ± 12.05	F = 0.078	0.925	90.47 ± 12.27
Cr (μmol/L)	75.04 ± 36.60	78.07 ± 39.72	71.56 ± 23.70	F = 0.940	0.349	74.04 ± 31.51
Total bilirubin (μmol/L)	36.83 ± 14.74	38.62 ± 17.21	34.10 ± 15.69	F = 0.868	0.387	35.89 ± 15.89
PPG before TIPS (mmHg)	25.41 ± 4.82	25.71 ± 5.49	25.84 ± 5.49	F = 0.073	0.929	25.71 ± 5.31
PPG after TIPS (mmHg)	9.52 ± 2.28	9.97 ± 3.04	10.13 ± 2.81	F = 0.530	0.590	9.95 ± 2.75
Deaths in 24 mo, *n* (%)	3 (9.4)	4 (11.4)	10 (14.3)	$X^2 = 0.517$	0.772	17 (13.7)
HCC in 24 mo, *n* (%)	2 (6.3)	2 (5.7)	7 (10)	$X^2 = 0.712$	0.700	11 (9.3)
HE in 24 mo, *n* (%)	6 (18.8)	8 (22.9)	18 (25.7)	$X^2 = 0.770$	0.681	32 (25.4)

MELD: Model for end-stage liver disease; TIPS: Transjugular intrahepatic portosystemic shunt; PPG: Portal pressure gradient; HE: Hepatic encephalopathy; HCC: Hepatocellular carcinoma; eGFR: Estimated glomerular filtration rate; TAF: Tenofovir alafenamide fumarate; TDF: Tenofovir disoproxil fumarate; ETV: Entecavir.

Twelve and twenty-four months after surgery, the incidence of liver cancer was 3.4% and 7.3% in the TAF group, 3.6% and 7.3% in the TDF group, and 6.1% and 11.3% in the ETV group, respectively (log rank $\chi^2 = 0.77$, $P = 0.681$) (Figure 1C). During the follow-up, liver cancer occurred in 11 patients, with a median time to occurrence of 11 (8, 14) mo (TAF *vs* TDF *vs* ETV, 2/2/7).

Child-Pugh score and constituent ratio before and after TIPS in three groups

Child-Pugh score showed no significant differences in the three groups before and 12 mo after surgery ($P > 0.05$). Twenty-four months after surgery, Child-Pugh score in the TAF group was 6.97 ± 0.86, which was lower than those in the TDF group (7.49 ± 0.82; $t = -2.52$, $P = 0.014$) and ETV group (7.64 ± 1.17; $t = -2.92$, $P = 0.004$) (Figure 2).

Twenty-four months after surgery, the constituent ratio of Child-Pugh stage A/B/C (60.8/34.8/4.4) in the TAF group was improved compared with that (28.1/56.3/15.6) before surgery ($\chi^2 = 6.47$, $P = 0.039$), but the constituent ratio of Child-Pugh stage A/B/C in the TDF/ETV group presented no difference from that before surgery ($P > 0.05$) (Table 2).

MELD score before and after surgery in three groups

No statistically significant differences were found in MELD score before and 12 mo after surgery in the three groups ($P > 0.05$). Twenty-four months after surgery, MELD score in the TAF group was 9.72 ± 1.5, which was lower than those in the TDF group (10.74 ± 2.33; $t = -2.09$, $P = 0.040$) and ETV group (10.97 ± 2.17; $t = -2.93$, $P = 0.004$) (Figure 3).

eGFR change before and after surgery in three groups

In the three groups, eGFR change showed no significant differences 3 mo after surgery

Table 2 Child-Pugh constituent ratio

	Baseline	1 mo	3 mo	6 mo	12 mo	24 mo
TAF, *n* (%)	9/18/5 (32)	4/23/5 (32)	3/23/6 (32)	6/22/4 (32)	7/18/2 (27)	14/8/1 (23)
Child-Pugh A/B/C (%)	28.1/56.3/15.6	12.5/71.9/12.7	12.7/74.6/15.6	18.8/68.8/12.5	25.9/66.7/7.4	60.9/34.8/4.3
TDF(*n*)	14/17/4 (35)	4/25/6 (35)	4/23/8 (35)	7/21/5 (33)	6/20/3 (29)	7/11/3 (21)
Child-Pugh A/B/C (%)	40.0/48.6/11.4	11.4/71.4/12.0	11.4/65.8/22.8	21.2/63.6/15.2	20.7/68.9/10.4	33.3/52.4/14.3
ETV, *n* (%)	21/37/12 (70)	9/47/13 (69)	6/47/16 (69)	11/44/11 (66)	11/42/6 (59)	11/24/5 (40)
Child-Pugh A/B/C (%)	30.3/52.9/17.1	13.1/68.1/18.8	8.7/68.1/23.2	16.7/66.6/16.7	18.6/71.2/10.2	27.5/60.0/12.5

TAF: Tenofovir alafenamide fumarate; TDF: Tenofovir disoproxil fumarate; ETV: Entecavir.

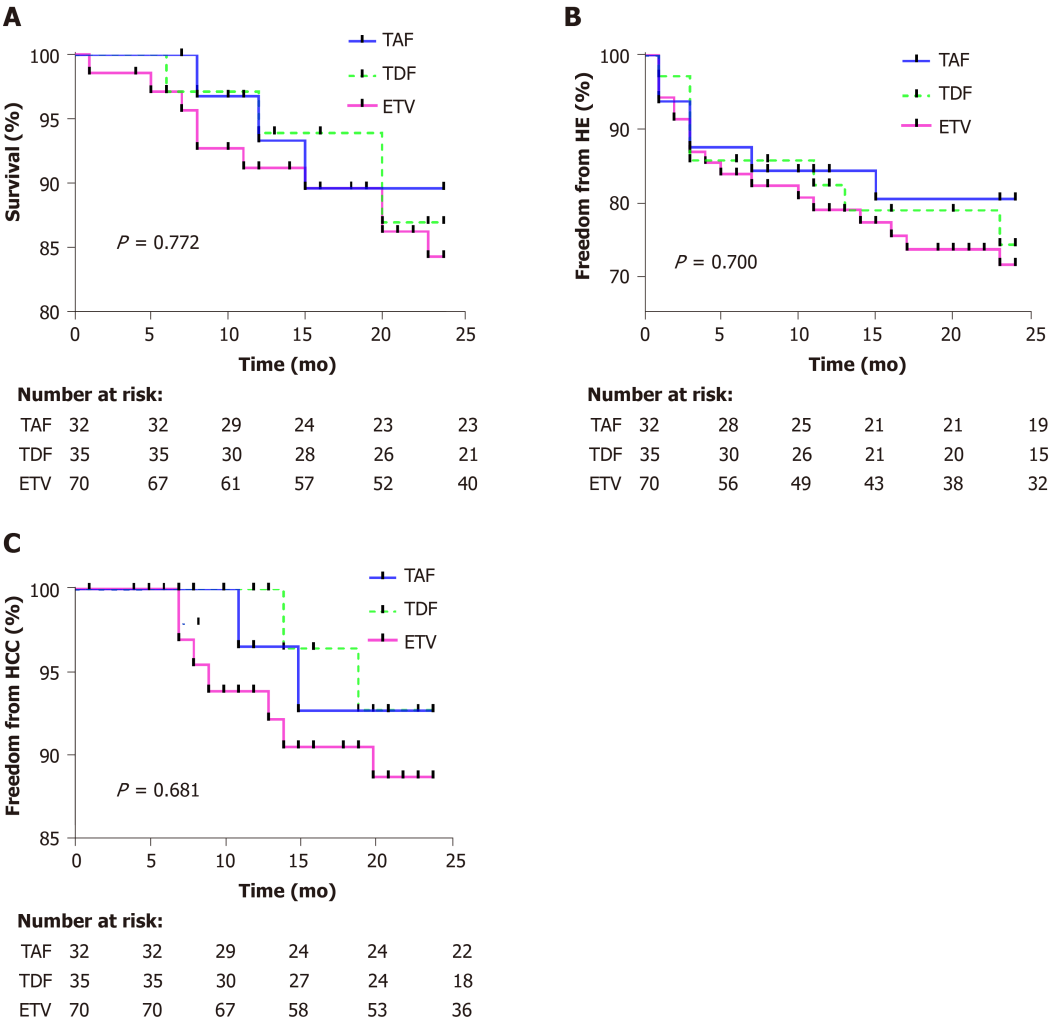


Figure 1 Overall survival, hepatic encephalopathy free survival, and hepatocellular carcinoma free survival rate in tenofovir alafenamide fumarate, tenofovir disoproxil fumarate, and entecavir groups. A: Survival; B: Freedom from hepatic encephalopathy; C: Freedom from hepatocellular carcinoma. HCC: Hepatocellular carcinoma; HE: Hepatic encephalopathy; TAF: Tenofovir alafenamide fumarate; TDF: Tenofovir disoproxil fumarate; ETV: Entecavir.

($P > 0.05$). Six months after surgery, eGFR level in the TAF group was 99.72 ± 11.52, which was higher than that in the ETV group (91.24 ± 12.60; $t = 3.24$, $P = 0.002$), but not significantly different from that in the TDF group (94.97 ± 12.52; $t = 1.61$, $P = 0.112$). Twelve months after surgery, eGFR level in the TAF group was 103.44 ± 13.02, which was higher than those in the TDF group (94.11 ± 12.34; $t = 3.08$, $P = 0.004$) and ETV group (90.03 ± 11.04; $t = 5.37$, $P < 0.001$). Twenty-four months after surgery, eGFR level in the TAF group was 104.41 ± 12.54, which was higher than those (93.54 ± 8.97) in the TDF (93.54 ± 8.97) and ETV (89.96 ± 9.86) groups ($F = 21.57$, $P < 0.001$) (Figure 4).

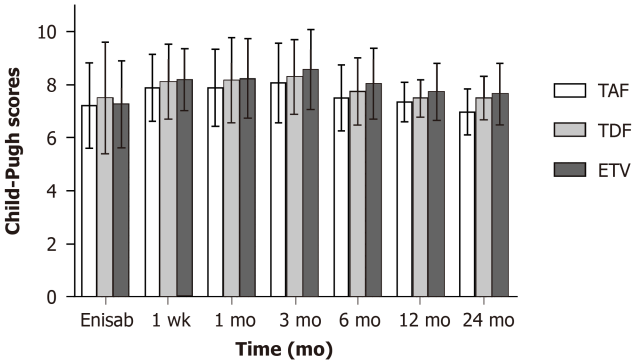


Figure 2 Preoperative and postoperative Child-Pugh scores and ratios in the three patient groups. TAF: Tenofovir alafenamide fumarate; TDF: Tenofovir disoproxil fumarate; ETV: Entecavir.

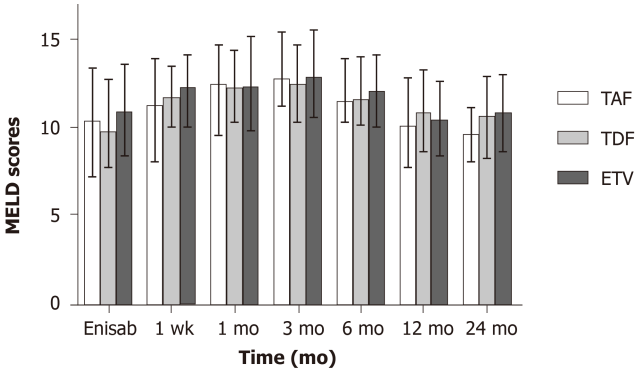


Figure 3 Changes in preoperative and postoperative model for end-stage liver disease scores in the three patient groups. MELD: Model for end-stage liver disease; TAF: Tenofovir alafenamide fumarate; TDF: Tenofovir disoproxil fumarate; ETV: Entecavir.

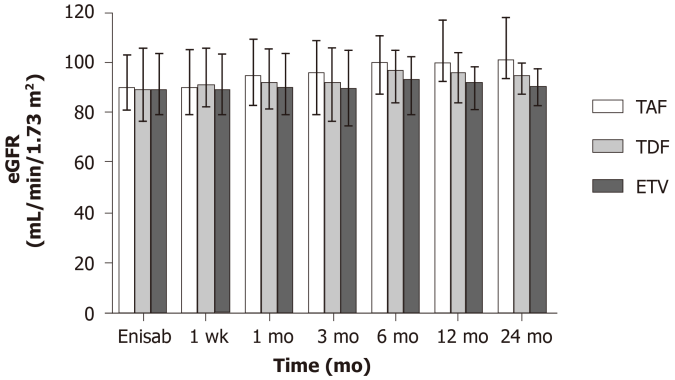


Figure 4 Changes in preoperative and postoperative estimated glomerular filtration rates in the three patient groups. eGFR: Estimated glomerular filtration rate; TAF: Tenofovir alafenamide fumarate; TDF: Tenofovir disoproxil fumarate; ETV: Entecavir.

DISCUSSION

As a country with a high burden of hepatitis B, China has about 86 million cases of HBV infection, ranking the first in the world. Currently, there are about 30 million CHB patients, and 390000 deaths due to hepatitis B-related complications such as liver cirrhosis and liver cancer every year[8,9]. Hepatitis B cirrhosis complicated with portal hypertension is characterized by persistent HBV replication and aggravated liver inflammation and fibrosis. Without timely control, it may progress to severe post-cirrhosis complications such as esophagogastric variceal bleeding, HE, intractable hydrothorax and ascites, and even hepatic failure and liver cancer, leading to death. Consequently, how to control portal hypertension, improve liver functional reserve, and reduce the incidence of hepatic failure and liver cancer in such patients is the

focus of current clinical attention. TIPS is an effective minimally invasive interventional method for the treatment of portal hypertension-related complications. It is worthy of further clinical exploration on the management of patients with hepatitis B cirrhosis using individualized antiviral therapy after surgery. Considering the fact that there is no report on the clinical efficacy of antiviral therapy for patients with hepatitis B cirrhosis after TIPS, we believe that this study has appreciated clinical reference value for the selection of anti-HBV drugs in such patients.

CHB is one of the main causes of HCC, cirrhosis-related complications, and liver-related death all over the world[10]. Chronic HBV infection is mainly treated by maximizing the long-term inhibition of HBV replication, relieving the inflammation and necrosis of hepatocytes and fibrous hyperplasia of the liver, delaying and reducing the occurrence of hepatic failure, decompensated cirrhosis, HCC, and other complications, improving the quality of life of the patients, and prolonging their survival time[11-13]. Antiviral therapy is a long-term or even lifelong process. The efficacy and safety of drugs are important factors that clinicians and patients need to consider. Previous studies have shown that in CHB patients with initial treatment, the incidence of drug resistance 5 years after ETV therapy is about 1.2%[14,15], while the clinical drug resistance of TDF and TAF has not been definitely reported[16,17]. Moreover, these three drugs are the first choice of oral antiviral drugs recommended by international and domestic guidelines, characterized by high barrier to resistance, strong effect, and high safety. In this study, all the included patients were those with hepatitis B cirrhosis after TIPS, and treated with the three antiviral drugs. During follow-up, the percentage of complete virological response of HBV DNA at 3, 6, and 12 mo after surgery was 100%, without breakthrough in virology. We believe that after continuous antiviral therapy, complete virological response can be achieved, inflammatory activity of the liver can be reduced or even become static, liver fibrosis can be reversed, and the regeneration ability of residual hepatocytes can be enhanced, which can further improve the liver functional reserve of the patients. In this study, TAF showed a better effect in improving the liver functional reserve of patients with hepatitis B cirrhosis at 24 mo after TIPS than TDF and ETV, the potential mechanism of which may be attributed to the stronger antiviral effect and ALT normalization ability of TAF. Previous global phase III clinical studies 108/110 on TAF[18,19] showed that compared with TDF, CHB patients treated with TAF could achieve a higher proportion of ALT normalization at both the 48th and 96th weeks. Moreover, an increasing number of real-world studies[20-22] demonstrated that about 30% of CHB patients receiving long-term ETV therapy failed to achieve complete virological response (the lower limit of detection of HBV DNA was 20 IU/mL), while most patients could achieve complete virological response after switching to TAF therapy. All these studies suggest that TAF has more prominent advantages in antiviral ability and/or ALT normalization. In our center, the lower limit of detection of HBV DNA was 100 IU/mL, which could not be used to accurately identify the CHB population with a low level of HBV DNA replication. However, the above findings may still have certain reference value for antiviral therapy in patients with hepatitis B cirrhosis complicated with portal hypertension after TIPS.

In fact, for patients with cirrhosis, especially for those with decompensated cirrhosis, liver transplantation is the only way to remove the lesion and radically cure cirrhosis. However, transplantation is rarely conducted in Asia. Especially in China, fewer patients can receive liver transplantation because of the limitation in funds and liver sources. For patients with hepatitis B cirrhosis, effective etiological treatment combined with control of portal hypertension and improvement of liver functional reserve is the key to long-term survival of patients with decompensated hepatitis B cirrhosis who cannot accept liver transplantation. TIPS is the only minimally invasive method for relieving portal hypertension. Previous clinical study[1] in our center suggested that the use of an 8-mm stent during TIPS could not only effectively shunt, but also relieve portal pressure. Postoperatively, the liver functional reserve was affected by shunt in a short time, but the special stent with an inner diameter of 8 mm had limited shunt. The liver functional reserve could be restored to the preoperative level 12 mo after surgery. Twenty-four months after surgery, the liver functional reserve of patients with hepatitis B cirrhosis was better than that of patients with alcohol-induced and immune cirrhosis, which may be related to the effective etiological treatment. Based on the above studies, the clinical data of patients with hepatitis B cirrhosis after TIPS treated with different antiviral drugs were retrospectively analyzed in this study. Before this study, many studies[23-27] in the past 2 years have explored the effects of different antiviral drugs on the long-term occurrence of HCC in patients with CHB, most of which are retrospective cohort studies, and some were systematic reviews and meta-analyses, with non-unified

conclusions. However, it can be concluded that nucleotide drugs (represented by TFV) are equal or superior to nucleoside analogues (represented by ETV) in the long-term risk of HCC, especially in some patients with high-risk factors for HCC, such as compensated cirrhosis or decompensated cirrhosis. In this study, it was found that during the follow-up, the incidence of HE and liver cancer as well as survival rate had no statistical significance among the three groups. We believe that it was related to the small number of patients enrolled in this study and short follow-up time. Because TAF has not been clinically applied in China for a long time, the long-term clinical outcomes of the three different antiviral drugs in the treatment of patients with hepatitis B cirrhosis after TIPS need to be further clarified by expanding the sample size and prolonging the observation time.

According to the Chinese guidelines for TIPS operation in 2019[28], TIPS can effectively improve glomerular filtration rate, increase renal blood flow, and reduce serum creatinine and aldosterone levels. The eGFR of all the patients included in this study was improved at varying degrees 6 mo after surgery. We believe that the short-term changes in renal function are related to the changes in systemic hemodynamics after TIPS, and effective control of portal pressure can improve renal blood supply and glomerular filtration rate. The safety of TAF for the bone and kidney has been proved to be higher than that of TDF in previous global phase III clinical studies 108/110[18, 19]. Additionally, the 2018 AASLD HBV guidelines[11] suggest no advantages or disadvantages between ETV and TDF for the risk of long-term renal and skeletal complications. Compared with TDF, TAF is associated with a lower proportion of patients with skeletal and renal abnormalities. Moreover, in the 2017 EASL guidelines [12], TAF or ETV is superior to TDF in CHB patients > 60 years old with bone diseases and renal changes. With the same baseline, eGFR of the TAF group was better than the other two groups 12-24 mo after surgery in this study. We believe that in the limited follow-up time, there were differences among the three drugs in the treatment of patients with hepatitis B cirrhosis after TIPS. TAF may have a smaller effect on the renal function of patients with decompensated hepatitis B cirrhosis than the other two drugs, which further indicates that TAF may have a smaller effect on the renal function of patients with CHB cirrhosis. Through clinical practice, we believe that patients with hepatitis B cirrhosis after TIPS can benefit from long-term treatment by active antiviral therapy and choosing TAF, which has a smaller effect on renal function and can potently inhibit viruses.

This study has several limitations. First, this is a retrospective cohort study. Although most indexes in different treatment groups were consistent at the baseline in this study, there were still potential unpredictable biases. Second, in this study, HBeAg status, HBsAg titer level, and ALT that may affect the long-term prognosis were not recorded and analyzed. Finally, the time from the approval of TAF in China to its clinical application is still short, and the number of patients receiving TAF therapy is relatively small. Therefore, the sample size of this study is small, and we need to further expand the sample size, and use propensity score matching statistical method to avoid excessive biases and baseline mismatch as much as possible. This study is continuing to determine whether the beneficial effect of TAF will last for a long time, and we will also conduct a longer-term clinical observation on the antiviral therapy in patients with hepatitis B cirrhosis after TIPS.

In our center, the operation and clinical management were performed by the same group of doctors. Through clinical practice, we believe that improving the long-term survival rate of patients with hepatitis B cirrhosis after TIPS involves a complex situation, which is determined by a large number of non-hemodynamic factors. Age, degree of renal failure, chronic inflammation, urease-producing intestinal bacteria, bacterial translocation and malnutrition/atrophy are other very important factors in the regulation of the treatment. Early initiation of antiviral therapy and optimization of antiviral therapy are also important factors. In conclusion, compared with TDF and ETV, TAF has significant advantages in the improvement of liver functional reserve and eGFR. The difference in the long-term effect of TAF on HCC occurrence needs further observation and clarification.

CONCLUSION

At 24 mo after surgery, compared with TDF and ETV, TAF has significant advantages in the improvement of liver functional reserve and eGFR. The difference in the long-term effect of TAF on HCC occurrence needs further observation and clarification.

ARTICLE HIGHLIGHTS

Research background

Hepatitis B cirrhosis complicated with portal hypertension is characterized by persistent hepatitis B virus (HBV) replication and aggravated liver inflammation and fibrosis. Without timely control, it may progress to severe post-cirrhosis complications such as esophagogastric variceal bleeding, hepatic encephalopathy (HE), intractable hydrothorax and ascites, and even hepatic failure and liver cancer, leading to death. Consequently, how to control portal hypertension, improve liver functional reserve, and reduce the incidences of hepatic failure and liver cancer in such patients is the focus of current clinical attention. Previous clinical study in our center suggested that the use of an 8-mm stent during transjugular intrahepatic portosystemic shunt (TIPS) could not only effectively shunt, but also relieve portal pressure. Twenty-four months after surgery, the liver functional reserve of patients with hepatitis B cirrhosis was better than that of patients with alcohol-induced and immune cirrhosis. Based on the above studies, the clinical data of patients with hepatitis B cirrhosis after TIPS treated with different antiviral drugs were retrospectively analyzed in this study.

Research motivation

TIPS is an effective minimally invasive interventional method for the treatment of portal hypertension-related complications. It is worthy of further clinical exploration on the management of patients with hepatitis B cirrhosis using individualized antiviral therapy after surgery. Considering the fact that there is no report on the clinical efficacy of antiviral therapy for patients with hepatitis B cirrhosis after TIPS, we believe that this study has appreciated clinical reference value for the selection of anti-HBV drugs in such patients.

Research objectives

To explore the clinical efficacy of the three antiviral drugs through this retrospective study, so as to provide reference for antiviral treatment of patients with chronic hepatitis B (CHB) cirrhosis after TIPS.

Research methods

The clinical data of 137 patients with hepatitis B-related cirrhosis with portal hypertension after receiving TIPS at our center between March 2016 and December 2020 were analysed retrospectively. According to different anti-viral drugs, the patients were divided into entecavir (ETV) (n = 70), tenofovir alafenamide fumarate (TAF) (n = 32), and tenofovir disoproxil fumarate (TDF) (n = 35) groups. The cumulative incidence of HE and hepatocellular carcinoma (HCC), survival, and changes in hepatic reserve function and glomerular filtration rate in patients treated with different antiviral drugs within 24 mo after surgery were investigated.

Research results

At 24 mo after surgery, compared with TDF and ETV, TAF had significant advantages in the improvement of liver functional reserve and estimated glomerular filtration rate (eGFR).

Research conclusions

Through clinical practice, compared with TDF and ETV, TAF has significant advantages in the improvement of liver functional reserve and eGFR. The difference in the long-term effect of TAF on HCC occurrence needs further observation and clarification. The author's team believes that improving the long-term survival rate of patients with hepatitis B cirrhosis after TIPS involves a complex situation, which is determined by a large number of non-hemodynamic factors. Early initiation of antiviral therapy and optimization of antiviral therapy are important factors. Age, degree of renal failure, chronic inflammation, urease-producing intestinal bacteria, bacterial translocation, and malnutrition/atrophy are other very important factors in the regulation of the treatment.

Research perspectives

The difference in the long-term effect of TAF on HCC occurrence needs further observation and clarification.

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ORIGINAL ARTICLE

Observational Study

Assessing disease activity using the pediatric Crohn's disease activity index: Can we use subjective or objective parameters alone?

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Abstract

BACKGROUND

The pediatric Crohn's disease activity index (PCDAI) is used as a standard tool to assess disease activity in clinical trials for pediatric Crohn's disease.

AIM

To examine which items on the PCDAI drive assessment of disease activity, and how subgroups of subjective and objective items reflect change in disease state over time.

METHODS

Selective raw data from three prospectively collected datasets were combined, including 703 children with full PCDAI data at baseline, at 3-mo (Q1, n = 670), and 1-year (Q4, n = 474). Change in individual PCDAI scores from baseline to Q1 and to Q4 were examined using the non-weighted PCDAI.

RESULTS

Abdominal pain, well-being, weight, and stooling had the highest change scores over time. Objective indicators including albumin, abdominal exam, and height velocity followed. Change scores for well-being and abdominal exam did not explain significant variance at Q1 but were significant predictors at Q4 ($P < 0.001$ and $P < 0.05$). Subjective and objective subgroups of items predicted less variance

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(18% and 22%) on total PCDAI scores at Q1 and Q4 compared to the full PCDAI, or a composite scale (both 32%) containing significant predictors.

CONCLUSION

Although subjective items on the PCDAI change the most over time, the full PCDAI or a smaller composite of items including a combination of subjective and objective components classifies disease activity better than a subgroup of either subjective or objective items alone. Reliance on subjective or objective items as stand-alone proxies for disease activity measurement could result in misclassification of disease state.

Key Words: Crohn's disease; Pediatric Crohn's disease activity index; Patient reported outcome measurement; Disease activity; Clinical trials; Pediatric

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Core Tip: The pediatric Crohn's disease activity index (PCDAI) is commonly used to assess disease activity in clinical trials. The PCDAI is a multi-item index incorporating subjective (*e.g.*, patient well-being) and objective (*e.g.*, laboratory tests) items. In response to a call from the Food and Drug Administration our team reexamined functioning of this index. Although subjective items on the PCDAI changed the most over time, the full PCDAI or a smaller composite of items that includes both subjective and objective components better classifies disease activity. Use of subjective or objective items on their own may result in misclassification of disease state.

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INTRODUCTION

The pediatric Crohn's disease activity index (PCDAI) has been the standard tool to assess clinical disease activity and response to treatment in clinical trials of pediatric Crohn's disease (CD) for the past two decades[1-4]. The PCDAI was developed by a group of clinicians and focuses on: (1) Subjective reporting of the degree of abdominal pain, stool pattern, and general well-being; (2) Extra-intestinal manifestations, such as fever, arthritis, rash, and uveitis; (3) Physical examination findings including abdominal pain, perirectal disease, extraintestinal manifestations, weight and height; and (4) Laboratory data, including hematocrit, erythrocyte sedimentation rate (ESR), and serum albumin[1] . This index has high inter-rater reliability, and good construct validity with physician global assessments of disease activity[1]. Validity has been further demonstrated in several studies assessing its psychometric properties[5-9].

Over time, concerns over both feasibility and short-term responsiveness to change in clinical status have arisen, due to the inclusion of perianal examination, as well as height velocity, which are not expected to change in a short-term (typically 8-12-wk period for induction of remission) clinical trial. These feasibility concerns are problematic in use of the PCDAI as the completion rate in routine practice of the PCDAI is approximately half (48% *vs* 98%)[10] that of the pediatric ulcerative colitis activity index (PUCAI), which does not include laboratory values, physical examination, or growth[11]. In response, other versions of the PCDAI have been developed, which have demonstrated increased feasibility: the abbreviated PCDAI[10,12,13], the Short PCDAI[10], and the modified PCDAI[14], but they have had lower face, construct and discriminant validity than the original PCDAI[5]. Subsequently, a mathematically weighted version (wPCDAI) that removed redundant and low feasibility items was established[5]. Despite multiple versions, there remain concerns around construct validity due to the mismatch between clinical symptoms relied upon in the wPCDAI and objective markers of inflammation as visualized endoscopically, or

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reflected *via* C-reactive protein or fecal calprotectin[15-17].

Traditionally, endpoints chosen for clinical trials have been based on multi-item indices[18,19], such as the PCDAI, which can incorporate symptoms, signs, laboratory tests, and endoscopic measures[20]. The PCDAI is still in use as an endpoint in many pediatric CD trials despite the feasibility concerns of these multi-item indices. As such, the United States Food and Drug Administration (FDA) sponsored the Gastroenterology Regulatory Endpoints and Advancement of Therapeutics (GREAT) workshops to reassess their use[20]. The FDA is seeking to redefine endpoints used in clinical trials, based on the need for objective measures of disease state. Additionally, recent regulatory guidance mandates that symptoms should be scored directly from the patient without a proxy[21]. This guidance recommends the development and use of Patient Reported Outcomes (PROs)[22-25] to measure clinical trial end-points, alongside objective markers of disease (such as endoscopic tools)[26] and health related quality of life[27,28].

The PCDAI is a composite of subjective items derived from a clinical observer, as well as objective items (laboratory studies), and as such it does not meet criteria set forth by the FDA for a PRO[22]. Although recent work has repurposed pieces of the PCDAI (ESR, stooling) with fecal calprotectin and C-reactive protein to develop a non-invasive index of mucosal healing in pediatric CD[29], there is no validated measure of subjective disease state in this population. A PRO for pediatric CD disease activity is still in the early stages of development[30] and is years away from implementation and use as an endpoint in clinical trials. Meanwhile, adult inflammatory bowel disease (IBD) trials are currently moving away from multi-item indices as endpoints and moving towards FDA guidance involving use of a PRO to measure functional components of disease alongside an objective marker of inflammation[31]. As such, as part of the GREAT workshops, the academic and pharmaceutical communities were asked to investigate and redefine the drivers of remission on the PCDAI given the urgent need for a PRO in pediatric CD.

In response to the FDA call we have set out to evaluate whether subgroups of items on the PCDAI could serve as an indicator of objective disease activity, or subjective disease activity until a PRO is ready for use. To date, post-hoc analyses of the PCDAI have not evaluated subcomponents that may serve as interim proxies of subjective or objective components, nor have they evaluated how individual items change over time. In the current study we evaluated the functioning of items on the PCDAI to determine how well subgroups of subjective and objective PCDAI items can capture disease activity.

MATERIALS AND METHODS

Ethics

Appropriate ethics approvals were attained across all participating sites at the original time of data collection. No additional ethics approvals were required for the purpose of this study.

Participants

Three prospectively collected datasets were merged and used for analysis, as follows.

The Registry[8]: The pediatric inflammatory bowel disease (IBD) collaborative research group registry. Data from Canada and the United States were collected on children (< 16 years old) newly diagnosed with IBD across 22 sites between 2002 and 2014. For children with CD, PCDAI, as well as clinical and demographic characteristics, physician global assessment of disease activity, and therapies were recorded at time of diagnosis or at study enrolment, which sometimes differed by up to a few weeks (hereinafter referred to as baseline), 30 d after diagnosis, and then quarterly. Children were not selected for inclusion based on disease severity. In the current study, data from baseline, first quarter (Q1), and at 1-year post diagnosis (Q4) were extracted for 414 children with PCDAI scores available.

The REACH study[4]: Clinical trial of infliximab use in pediatric CD. This randomized controlled trial of infliximab use included 112 children 6-17 years old who had moderate to severe CD (PCDAI > 30) at baseline. This trial involved a 10-wk induction phase, followed by a 1-year maintenance phase. Data at baseline, 10 wk (considered Q1), and 1-year (Q4) were extracted for the current study.

The IMAgINE trial[32]: Clinical trial of adalimumab use in pediatric CD. In this randomized controlled trial, 192 patients between 6 and 17 years old, with moderate to severe CD (PCDAI > 30) received open-label adalimumab at baseline and at 2 wk

before being randomized in blinded fashion at week 4 to high or low dose alternate weekly adalimumab maintenance for an additional 48 wk. Patients were stratified prior to randomization by week 4 responder status and prior exposure to infliximab. Data for the current study were extracted at baseline, 12 wk (Q1) and 52 wk post-treatment initiation (Q4).

Measures

The original version of the 11-item PCDAI (*i.e.*, non-weighted) was used in this study [1], as this was in keeping with how data were collected, scored, and analyzed in each of the original studies. The PCDAI was used to assess disease activity, with the acknowledgement that there is no current gold standard of disease activity in pediatric CD assessment. Severity for each item is assigned a score of 0 (normal), 5 (mild abnormality), or 10 (severe abnormality) except for hematocrit and ESR which are scored as 0, 2.5, or 5. A minimum total index score is zero, and a maximum score is 100. Remission was defined as a total PCDAI score ≤ 10, congruent with the REACH and IMAgINE trials[4,32].

Data acquisition

Relevant raw data were provided from each of the studies and were merged for group analysis. Basic demographic characteristics (age, gender, disease duration), and PCDAI scores at the above-mentioned time points were included. Physician Global Assessment (REACH, Registry), Subject and Parent Global Assessment (REACH), as well as IMPACT, a 35-item health related quality of life self-report measure for paediatric IBD patients[33] (REACH, Registry) were also provided, but were not included in the current analysis. Only data relevant to the proposed analyses were provided, thus, for further information on disease location, or types of treatment, please see the original manuscripts for the REACH[4] and IMAgINE[32] trials.

Inclusion criteria

Only participants with complete PCDAI data at baseline (i.e. had a score for each item on the questionnaire) were included. Thus, sample sizes for clinical trial datasets differ from the original reports, which used different missing data rules (*e.g.*, last value carried forward) as appropriate to the methodology of each respective trial. Sample sizes differ when examining change in PCDAI scores over time, as not all participants had complete data at each respective timepoint. Data were determined to be missing at random (*i.e.* did not attend scheduled follow-up appointment, some clinical or laboratory data parameters not collected due to established PCDAI feasibility issues).

Statistical analysis

Differences in demographic characteristics and baseline scores for participants with and without complete PCDAI data at each timepoint were compared using Independent samples Mann-Whitney U tests. PCDAI change scores were calculated by subtracting the score at baseline from the score at each follow-up time point (either Q1 or Q4, as appropriate). Repeated measures general linear models were run, which examined the mean change from baseline to Q1 and from baseline to Q4 for individual PCDAI items. Fisher's least significant difference tests for pairwise comparisons were run within the general linear model to examine the rank order of change scores across items. Multivariate linear regressions were run to examine the relative contribution of individual PCDAI change scores (entered in weighted units) from baseline to total disease activity at Q1 and Q4. Unstandardized beta values are reported. Subsequent models were run to examine the relative performance of subjective and objective item change scores that significantly contributed to the overall score at Q1 and Q4 follow-up, as well as models looking at only those items that significantly contributed to the overall score at each follow-up time point.

Serial receiver operating characteristic curves (ROC) were run to determine the optimal total score cut-point that differentiates between remitters and non-remitters for the two subscales containing subjective items and objective items. The best cut-off score maximized sensitivity and specificity, corresponding to the upper left shoulder of the ROC curve. The cut-points were then used to examine how well subjective and objective scales alone would similarly classify patients as achieving remission (remitters) *vs* non-remitters compared to the full PCDAI scale score.

All analyses were carried out using SPSS V24.0 (Chicago, IL, United States).

RESULTS

A total of 703 children, with full PCDAI data at baseline, were included in the current study (Table 1). Patients enrolled in the Registry were all at diagnosis, of varying disease severity, and treated per physician dictate, not by protocol. Patients enrolled in the REACH and IMAGINE studies had moderate to severe disease activity at study entry, had a mean disease duration of approximately two years since diagnosis, and were treated by the clinical trial protocol. Basic demographic information, including age, disease duration, and mean PCDAI scores, are presented separately for each dataset as the Registry sample was significantly different than each of the trials with respect to age at baseline (younger), age at diagnosis (younger), and baseline PCDAI (lower) (all $P < 0.001$). The data were then merged and analyzed together for all subsequent analyses, as the goal with merging the data was to have a diverse and representative sample. Sample sizes differ for each follow-up time point as some participants had incomplete PCDAI data at those follow-ups. There were some differences between those who had full PCDAI data at Q1 ($n = 670$), as participants who did not have full Q1 data ($n = 39$) were somewhat older (13.5 ± 2.3 *vs* 12.5 ± 2.8 years, $P = 0.059$), but were significantly younger at diagnosis (10.2 ± 2.9 *vs* 11.5 ± 2.8 years, $P = 0.004$), and had higher baseline PCDAI scores (42.1 ± 9.6 *vs* 34.9 ± 13.7, $P < 0.001$). Participants who did not have full PCDAI data at Q4 ($n = 235$) were significantly younger (12.0 ± 2.7 *vs* 12.8 ± 2.8, $P < 0.001$) and had lower baseline PCDAI scores (32.3 ± 14.7 *vs* 36.89 ± 12.7, $P < 0.001$) compared to those with full PCDAI data at Q4 ($n = 474$). There were no differences in gender distribution between those who did and did not have complete data at each time point (both $P > 0.53$).

The mean absolute change of individual PCDAI items was examined over time for individuals who had complete PCDAI data from baseline to Q1 ($n = 670$), and from baseline to Q4 ($n = 474$) (Figure 1). Of note is that accurate comparisons of mean change cannot be made between hematocrit or ESR and the other PCDAI items as they are scored on 5-point *vs* the 10-point scale of the other PCDAI items. The mean change of individual items was similar at both Q1 and Q4. Abdominal pain, well-being, weight, and stooling had a larger degree of change from baseline to Q1 and Q4 in comparison to the other items ($P < 0.05$). Other objective indicators of disease activity followed, with albumin, abdominal exam, height velocity, and ESR being within the next four most highly ranked items showing similar degrees of absolute change over time to both follow-up time points.

Two linear regression analyses were run to examine the contribution of individual PCDAI change scores to disease activity at Q1 and Q4 (Table 2). At Q1, abdominal pain, stooling, ESR, albumin, weight, height velocity, and perirectal examination were all significant predictors of disease activity (all $P < 0.05$). Three additional models were run: (1) Subjective; (2) Objective; or (3) A composite scale of PCDAI items that were significant predictors in the full model. All the models were significant (all $P < 0.001$), however, the proportion of variance accounted for by the PCDAI questions included was highest for the total and composite scales (both $R^2 = 0.32$), where less variance was accounted for on the models including only the subjective ($R^2 = 0.18$) and objective ($R^2 = 0.21$) items. Similar results were found at Q4, except that the PCDAI change scores from baseline to Q4 were also significant for well-being ($P < 0.001$), abdominal exam ($P < 0.05$), and extraintestinal manifestations ($P < 0.05$). The three additional models also all accounted for a significant amount of variance (all $P < 0.001$), with the subjective scale at this time point also including well-being, and the objective scale included abdominal exam and extraintestinal manifestations, as previously discussed. Similar to Q1, more variance was accounted for by the full and composite models (both $R^2 = 0.45$), than for the subjective ($R^2 = 0.22$) or objective ($R^2 = 0.36$) models.

ROC curves were run to examine how well these sub-scales of subjective and objective items would function on their own, as a proxy to measure disease activity. Items included on the subjective and objective subscales were made based on which item change scores significantly predicted disease activity at the first follow-up (Q1) as this is a similar timeframe to many clinical trials and corresponds with the degree of clinical change observed during a feasible assessment time frame. Cut-points to define remission were defined at Q1 ($n = 670$), using a composite of the significant subjective items (abdominal pain and stooling) (Figure 2A) and objective items (ESR, albumin, weight, height velocity, and perirectal examination) (Figure 2B). A cut-point of ≤ 2.5 (out of a maximum score of 20) best defined remission for the subjective composite, based on an area under the curve of 0.84 (95%CI: 0.80-0.87), with a sensitivity of 74% and a specificity of 83%. For the objective composite, a cut-point of ≤ 3.75 (out of 45) defined remission, with an area under the curve of 0.85 (95%CI: 0.82-0.88), sensitivity of 71% and specificity of 78%.

Table 1 Descriptive statistics

	Registry (*n* = 414[1])		REACH (*n* = 101[1])		IMAgINE (*n* = 188[1])	
	Range	Median (IQR)	Range	Median (IQR)	Range	Median (IQR)
Age at baseline	1.5-16.1	12.2 (3.6)	6.0-17.8	13.8 (3.2)	6-17.0	14.0 (4.0)
Age at diagnosis	1.5-15.9	12.2 (3.6)	4.8-16.6	11.8 (4.1)	2.4-16.4	10.6 (3.2)
Gender (female), *n* (%)	180 (43.5)		40 (39.6)		82 (43.6)	
	Range	mean ± SD	Range	mean ± SD	Range	mean ± SD
PCDAI BL	0-82.5	31.39 ± 15.34	25-62.5	41.14 ± 8.18	7.5-62.5	40.97 ± 7.48
PCDAI Q1	0-52.5	10.25 ± 9.75	0-40	9.77 ± 8.55	0-57.5	16.78 ± 12.64
PCDAI Q4	0-50.0	8.58 ± 9.21	0-70	12.07 ± 14.10	0-70	19.56 ± 16.34

[1]The baseline characteristics describe participants with full pediatric Crohn's disease activity index (PCDAI) data (*i.e.* had individual PCDAI scores collected) at baseline. Q1 PCDAI scores describe participants with full baseline and Q1 PCDAI data available (*n* = 414, 97, and 159 participants for the Registry, REACH, and IMAgINE datasets respectively). Q4 PCDAI scores describe participants with full baseline and Q1 PCDAI data available (*n* = 216, 75, and 183 participants for the Registry, REACH, and IMAgINE datasets respectively). PCDAI: Pediatric Crohn's disease activity index.

Remission status at Q1 and Q4 was examined using subscales of subjective and objective items with the cut-points identified above to examine the comparability of using subscales compared to the full PCDAI (Table 3). This table demonstrates the proportion of patients that would be classified the same or different as the original PCDAI scale by using subscale scores only, thus either in remission ("remitters") or having active disease ("non-remitters"). At Q1, of those classified as in remission using the full PCDAI, only 73.8% would be classified as in remission using the subjective subscale, and thus 26.2% of patients would be classified incorrectly. Using the objective scale, 71.5% of patients deemed remitters using the full PCDAI would be classified as being in remission. For patients not in remission using the full PCDAI, 16.7% would be differently classified using the subjective scale, and 21.2% using the objective scale. At Q4 73.7% of those in remission according to the full PCDAI scale would be in remission based on the subjective subscale, and 74.5% would be in remission according to the objective subscale. For those with active disease at Q4, 77.5% would be similarly defined as having active disease using the subjective subscale, and 72.0% would have active disease using the objective subscale.

DISCUSSION

In response to the FDA call for reevaluation of subcomponents of the PCDAI, and through work generated through the GREAT-III workshops, we have evaluated items on the PCDAI to determine the suitability of these subjective components in assessing disease activity on their own–while we await the development of a PRO for pediatric CD that fulfills FDA guidance. The results of this study indicate that relying on these items, as they are currently measured (*i.e.*, clinician report, nominal variable scoring), will not suffice as a substitution for a robust PRO outcome measure. However, the results do indicate that subjective items such as abdominal pain and stooling in the short-term, and well-being in a longer-term measurement are adequate indicators of disease activity and response to treatment.

We examined both absolute change in scores over time, in addition to looking at what change scores explained unique variance in disease activity at a 3-mo and 1-year follow-up. Items that changed the most over this period were similar. Subjective items including abdominal pain, well-being, and stooling were consistently among the top four items that changed the most over these periods. The rank order of the remaining items varied across these two time points, although objective markers of disease activity including weight, albumin, and abdominal exam were amongst the next group of items that changed the most over time. Results were similar when looking at the unique contributions of the change in item scores to the follow-up total PCDAI score at each time point. However, well-being was not a unique predictor at the 3-mo follow-up, nor was abdominal exam. At the 1-year follow-up however, almost all the change scores from baseline to 1-year added unique variance to the total score, except for hematocrit.

Table 2 Linear regression analysis examining the contribution of pediatric Crohn's disease activity index change scores to Q1 and Q4 pediatric Crohn's disease activity index disease activity

	PCDAIΔ Q1 to BL (n = 670)			
	Full scale	Subjective	Objective	Composite
	β values	β values	β values	β values
1. Abdominal pain	0.57[b]	0.80[b]		0.65[b]
2. Stooling	0.42[b]	0.53[b]		0.45[b]
3. Well-being	0.13			
4. HCT	-0.09			
5. ESR	0.94[b]		1.27[b]	0.96[b]
6. Albumin	0.46[b]		0.41[b]	0.45[b]
7. Weight	0.30[b]		0.52[b]	0.32[b]
8. Height Velocity	0.50[b]		0.56[b]	0.48[b]
9. Abdominal exam	0.08			
10. Perirectal exam	0.47[b]		0.45[a]	0.48[b]
11. Extraintestinal	0.13			
R^2	0.32	0.18	0.21	0.32
	PCDAIΔ Q4 to BL (n = 474)			
1. Abdominal Pain	0.45[b]	0.65[b]		0.45[b]
2. Stooling	0.42[a]	0.52[b]		0.42[a]
3. Well-being	0.58[b]	0.87[b]		0.58[b]
4. HCT	-0.01			
5. ESR	1.32[b]		1.56[b]	1.32[b]
6. Albumin	0.81[b]		0.84[b]	0.81[b]
7. Weight	0.49[b]		0.63[b]	0.49[b]
8. Height velocity	0.45[b]		0.48[b]	0.45[b]
9. Abdominal exam	0.35[a]		0.83[b]	0.35[a]
10. Perirectal exam	0.78[b]		0.90[b]	0.78[b]
11. Extraintestinal	0.42[a]		0.67[a]	0.42[b]
R^2	0.45	0.22	0.36	0.45

[a]$P < 0.05$.

[b]$P \leq 0.001$. PCDAI: Pediatric Crohn's disease activity index; HCT: Hematopoietic cell transplantation; ESR: Erythrocyte sedimentation rate.

Dominant PCDAI items (*i.e.*, items that contributed significant, unique variance) were similar to previous work examining functioning of the PCDAI and the contribution of individual items to change in disease activity[9]. At the 3-mo follow-up, well-being, hematocrit, abdominal exam, and extraintestinal manifestations did not explain unique variance in the total PCDAI score. This is similar to previous work demonstrating the limited responsiveness of specific items on the PCDAI, where abdominal exam, height, and hematocrit were removed from the currently recommended weighted PCDAI as they were found to be redundant in predicting change in disease activity as measured by physician global assessment[5]. At the 1-year follow-up, change scores for all items contributed significant unique variance to the total PCDAI score except for hematocrit. The strength in the current work is the examination of change scores over a 1-year period, highlighting the composite nature of the PCDAI, and that the change in items such as height velocity, abdominal exam, and extraintestinal manifestations take a longer time to demonstrate statistically unique or meaningful change but are nonetheless important components of overall well-being and functioning in patients with CD.

Table 3 Correct and incorrect identification of remitters and non-remitters

Full PCDAI-remission status	Subjective-remission status		Objective-remission status	
	Remitters	Non-remitters	Remitters	Non-remitters
	n (%)	*n* (%)	*n* (%)	*n* (%)
Remitters at Q1 (*n* = 397)	292 (73.8)	105 (26.2)	284 (71.5)	113 (28.5)
Non-remitters at Q1 (*n* = 273)	46 (16.7)	227 (83.3)	58 (21.2)	215 (78.8)
Remitters at Q4 (*n* = 274)	202 (73.7)	72 (26.3)	204 (74.5)	70 (25.3)
Non-remitters at Q4 (*n* = 200)	45 (22.5)	155 (77.5)	56 (28.0)	144 (72.0)

Subjective subscale is composed of abdominal pain and stooling; Objective subscale is composed of erythrocyte sedimentation rate, albumin, weight, height velocity, and perirectal examination. Shaded cells = number of patients that would be incorrectly (or differently) classified as having active or inactive disease based on the subjective components alone (*e.g.*, At the Q1 time point, 397 patients were classified as being in remission, but based on the subjective only scoring, 292 (73.8%) of those patients would be classified as being in remission, while the other 26% would be classified as having active disease). PCDAI: Pediatric Crohn's disease activity index.

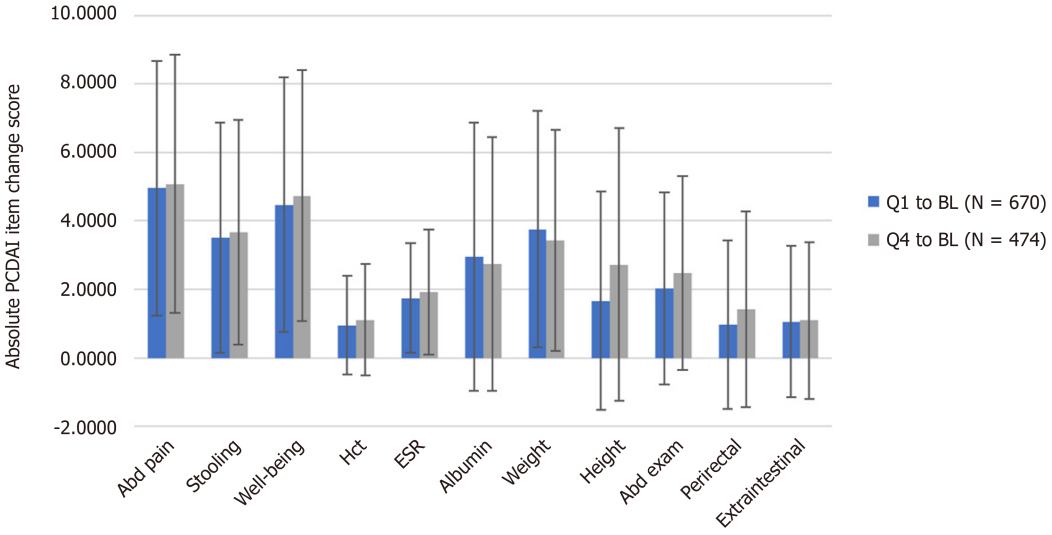


Figure 1 Mean change over time (from baseline to Q1 and Baseline to Q4). Hematocrit and erythrocyte sedimentation rate are scored on a 0-5-point scale, whereas all other items are scored on a 0-10 point scale.

Further examination of the subjective and objective subcomponents highlighted that change in both subcomponents over time does contribute significantly to disease activity. However, change in either subjective or objective groups of items on their own did not perform as well as the full-scale, or to a subgroup of questions (items that were significant unique predictors of total scores) that served as a composite measure of disease activity. If these subcomponents were used to classify patients into remission or active disease, a large portion of patients would be classified differently compared to using the full-scale. The outcomes at the 3-mo and 1-year assessments were similar, where approximately 25% of patients in remission with the full scale would be classified as having active disease with either the subjective or objective scales.

For patients with active disease, the percentage of patients misclassified ranged from about 16% to 22% with the subjective scale, whereas the range was 21% to 28% misclassified with the objective scale at both follow-up time points. Although the composite of items showed similar performance to the full PCDAI, the goal of this work was not to recommend or endorse a composite PCDAI measure as this work has already been completed[5,10], and our findings produced similar results as previously described. Rather, the focus of the current work around these smaller subgroups of items reflects the limited ability of either subjective or objective components alone, as measured on the PCDAI, to fully characterize disease activity.

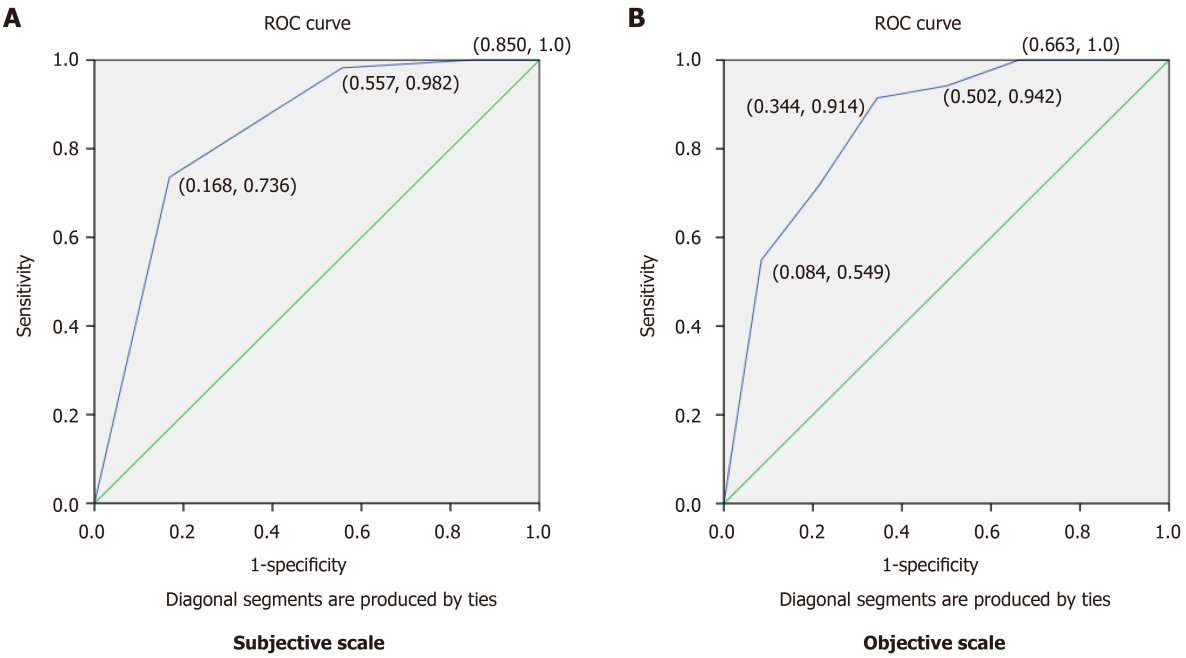


Figure 2 Receiver operator characteristic curve to identify the equivalent remission cut point of the subjective and objective scales. A: Subjective scale; B: Objective scale. ROC: Receiver operator characteristic.

Our study has several limitations, where the main limitation in the current work is the lack of "gold standard" measurement of disease activity beyond physician global assessment. The examination of the change in subjective and objective items on the PCDAI was compared over time but in relation to measurement of disease activity with the same measure at follow-up time points. Other work in trying to assess the psychometric properties of existing measures of disease activity in pediatric CD has found limited correlation with objective measurement of mucosal inflammation with the Simple Endoscopic Score for CD[15,16]. Additionally, work to identify biomarkers that accurately identify disease activity in pediatric CD is ongoing[34], but currently suggests that fecal calprotectin and C-reactive protein are reliable markers with utility in management of a patient's condition[35]. These indices also demonstrate limited correlation with C-reactive protein, and a poor correlation with fecal calprotectin[15,16]. Although there were some differences (younger at diagnosis, higher disease activity at baseline) between participants who had complete follow-up data at 1 year and those who did not, these differences were not considered problematic in the context of the current study. The objective of this study was to examine performance of the PCDAI rather than response to treatment, thus differences between those with and without complete data were not thought to be influential factors that would affect outcomes. In the absence of a universal gold standard measure, the current study is a first step in further understanding the functioning of subjective and objective assessment of disease activity as measured on the primary outcome measure used in clinical trials-the PCDAI.

Without a pediatric CD PRO ready for use, it is tempting to consider using measures already in existence to serve as an interim substitution. However, our study found that looking at subjective items alone in a large sample of patients with a range of disease activity is likely to misclassify many of these patients by relying on subjective items alone. Although subjective items on the PCDAI change the most over time, these items do not capture disease activity as well as a composite of PCDAI items containing both subjective and objective components, or in comparison to the full PCDAI scale. It is not surprising that a composite scale might outperform individual items given the protean nature of CD. This may be particularly relevant when looking over longer periods of time when changes in growth parameters may be more evident.

CONCLUSION

Our analysis highlights the limitations of relying only on subjective or objective items as currently measured on the PCDAI as stand-alone proxies for disease activity

measurement in pediatric patients with CD. Measuring improvements in health outcomes should be informed by evidence-based information produced by patients [36]. Where improvement in disease state may not reflect improvements in a patients well-being[37], our data reiterate that complementary subjective (*i.e.*, PROs)[28] and objective markers of disease[26] should be used to evaluate the outcomes of treatment.

ARTICLE HIGHLIGHTS

Research background

The pediatric Crohn's disease activity index (PCDAI) is a standard tool to assess disease activity in clinical trials for pediatric Crohn's disease. Over time, concerns over both feasibility and short-term responsiveness to change in clinical status have arisen. Based on feasibility concerns, and new guidance recommending that symptoms are scored directly from a patient, the PCDAI was reexamined.

Research motivation

In response to a call from the Food and Drug Administration our team reexamined functioning of this index.

Research objectives

The objective of this study was to examine which items on the PCDAI drive assessment of disease activity, and how subgroups of subjective and objective items reflect change in disease state over time.

Research methods

We retrospectively examined data from three completed studies – one registry study and two clinical trials involving pediatric patients with Crohn's disease. Data was collected at baseline, at 3-mo (Q1) and 1-year (Q4). Change in individual PCDAI scores from baseline to Q1 and to Q4 were examined using the non-weighted PCDAI.

Research results

Abdominal pain, well-being, weight, and stooling had the highest change scores over time. Objective markers of disease activity including weight, albumin, and abdominal exam were amongst the next group of items that changed the most over time. Subjective and objective subgroups of items predicted less variance on total PCDAI scores at Q1 and Q4 compared to the full PCDAI, or a composite scale containing significant predictors.

Research conclusions

Although subjective items on the PCDAI change the most over time, the full PCDAI or a smaller composite of items including a combination of subjective and objective components classifies disease activity better than a subgroup of either subjective or objective items alone. However, the results do indicate that subjective items such as abdominal pain and stooling in the short-term, and well-being in a longer-term measurement are adequate indicators of disease activity and response to treatment. Reliance on subjective or objective items as stand-alone proxies for disease activity measurement could result in misclassification of disease state.

Research perspectives

Subjective or objective items alone, as currently measured on the PCDAI, do not serve as a substitute for a robust patient-reported outcome measure. Complementary subjective (*i.e.*, patient reported outcome measures) and objective markers of disease should be used to evaluate the outcomes of treatment.

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ORIGINAL ARTICLE

Prospective Study

Naïve hepatitis B e antigen-negative chronic hepatitis B patients are at risk of carotid atherosclerosis: A prospective study

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Abstract

BACKGROUND

There is an increased risk of atherosclerosis in patients with chronic hepatitis C or human immunodeficiency virus, but there is scarce data on hepatitis B virus infection. The hypothesis of this study is that hepatitis B virus infection increases the risk of carotid plaques and subclinical atherosclerosis in naïve hepatitis B e antigen (HBeAg) negative subjects.

AIM

To assess the rate of carotid plaques and subclinical atherosclerosis in naïve HBeAg negative subjects in comparison with a cohort of healthy controls.

METHODS

Prospective case-control collaborative study conducted in two tertiary hospitals. Four hundred and two subjects prospectively recruited at the outpatient clinic were included from May 2016 to April 2017: 201 naïve HBeAg-negative hepatitis

B virus-infected [49 chronic hepatitis B (CHB) and 152 inactive carriers(ICs)] and 201 healthy controls. Anthropomorphic and metabolic measures, liver stiffness and carotid Doppler ultrasound were performed. Subclinical atherosclerosis was established on an intima-media thickness increase of ≥1.2 mm and/or the presence of carotid plaques. Normally distributed quantitative variables were compared with the Student *t* test and those with a non-normal distribution with the Mann-Whitney *U* test. Categorical variables were compared between groups using the χ^2 or Fisher exact test.

RESULTS

Carotid plaques were found more often in CHB (32.7%) than ICs (17.1%) or controls (18.4%) ($P = 0.048$). Subclinical atherosclerosis was also increased in CHB (40.8%) *vs*ICs (19.1%) or controls (19.4%) ($P = 0.003$). No differences in the risk of atherosclerosis were observed between controls and ICs. The factors independently associated with the presence of carotid plaques were age [odds ratio(OR) 1.43, $P < 0.001$] and CHB (OR 1.18, $P = 0.004$) and for subclinical atherosclerosis, age (OR 1.45, $P < 0.001$), CHB (OR 1.23, $P < 0.001$) and diabetes (OR 1.13, $P = 0.028$). In the subset of young subjects (< 50 years), carotid plaques (12.5% *vs* 1.1%, $P = 0.027$) and subclinical atherosclerosis (12.5% *vs* 2.2%, $P = 0.058$) were more frequent among CHB than ICs.

CONCLUSION

Untreated HBeAg-negative CHB is an independent risk factor for carotid plaques and subclinical atherosclerosis, while ICs present a similar risk to controls.

Key Words: Hepatitis B virus; Carotid plaques; Subclinical atherosclerosis; Cardiovascular risk; Endothelial dysfunction; Intima-media thickness

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Core Tip: This prospective case-control collaborative study aimed to assess whether chronic infection by hepatitis B was associated with risk of carotid plaques and subclinical atherosclerosis. Overall, 402 subjects were recruited, 201 naïve hepatitis B e antigen-negative hepatitis B virus-infected and 201 healthy controls Patients with hepatitis B e antigen-negative chronic hepatitis B presented a higher rate of carotid plaques than non-infected controls,but no differences were observed between controls and hepatitis B inactive carriers. These results suggest that hepatitis B infection may have a role as a cardiovascular risk factor in patients with chronic hepatitis B.

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INTRODUCTION

More than 257 million people worldwide are infected with hepatitis B virus (HBV)[1], and more than 780000 die each year due to the infection[2]. Chronic HBV infection is a dynamic condition that passes through several phases, being the hepatitis B e antigen (HBeAg)-negative form the most common in Western countries[1]. Currently, patients are classified as HBeAg-negative chronic hepatitis B (CHB) when they have increased HBV DNA and alanine aminotransferase (ALT) levels and liver fibrosis and/or necroinflammation or as HBeAg-negative chronic infection/inactive carriers (ICs)when they have low HBV DNA and normal ALT levels and associated with absent or mild liver damage[3].

were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. It was approved by the Ethics Committee of both hospitals (PR(AG)245/2015).

Informed consent statement: Informed verbal consent was obtained from all individual participants included in the study and recorded at the medical records.

Conflict-of-interest statement: Riveiro-Barciela M has received research grants from Gilead and served as speaker for Gilead and Grifols. Esteban R has received research grants from Gilead and has served as advisors for Gilead, Bristol-Myers Squibb and Novartis. Buti M has received research grants from Gilead and has served as advisors for Gilead, Bristol-Myers Squibb and Novartis. The rest of authors have no personal or financial conflicts of interest.

Data sharing statement: Technical appendix, and dataset available from the corresponding author at [mbuti@vhebron.net]. Participants gave informed verbal consent for data sharing.

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An increased risk of cardiovascular events has been associated with some viral infections like hepatitis C virus (HCV)[4] or human immunodeficiency virus (HIV)[5] as well as autoimmune diseases[6]. The cause of atherosclerosis in these patients is not fully explained by conventional risk factors, and endothelial dysfunction has been suggested as the underlying mechanism causing the early atherosclerotic process. This endothelial dysfunction is mainly associated with the persistent inflammatory state linked to these diseases (HCV, HIV and autoimmune diseases). In fact, eradication of HCV infection has shown a positive impact on carotid atherosclerosis[7]. Both the presence of carotid plaques or measurement of the intima-media thickness (IMT) are accepted and validated surrogate markers for early diagnosis of subclinical atherosclerosis leading to increased cardiovascular risk[8].

Chronic HBV infection has been associated with a propensity to mount proinflammatory immune reactions[9,10], including higher oxidative stress[11], that may predispose to a higher subclinical atherosclerosis.

The aim of this study was to assess whether the stage of HBeAg-negative chronic HBV infection impacts the presence of both carotid plaques and subclinical atherosclerosis. Another aim was to evaluate if the risk of both carotid plaques and subclinical atherosclerosis in HBeAg-negative patients differ to those of healthy controls.

MATERIALS AND METHODS

Patients

Two hundred and one patients with chronic HBV infection and naïve to antiviral therapy were prospectively recruited at the outpatient clinics of two tertiary hospitals (Di.Bi.M.I.S., University of Palermo, Italy and Vall d'Hebron Hospital, Spain) from May 2016 to April 2017. Inclusion criteria were hepatitis B surface antigen (HBsAg) positive for more than 6 mo, HBeAg-negative and no prior exposure to antiviral therapy. Exclusion criteria were previous cardiovascular events (acute myocardial infarction or ischemic stroke), liver transplantation, HCV, hepatitis D or HIV coinfection, history of hepatocellular carcinoma or evidence of liver disease of mixed etiology (autoimmune hepatitis, Wilson's disease, hemochromatosis, α1-antitriypsin deficiency). In addition, 201 healthy individuals matched for sex, age and body mass index were recruited as controls at the outpatient clinics from the same centers. In particular, no patient had a history of previous cardiovascular events, evidence of HBV infection (HBsAg and anti-HBc negative), HCV or HIV, or history of rheumatic or oncological disease. Importance of selection of naïve patients was crucial in view of the effect of antiviral therapy in both liver immunity and carotid plaques in subjects with HCV treated with direct-acting antivirals[7].

Naïve patients with HBV infection were classified into CHB and IC according to the recommendations of European Association for the Study of the Liver[3]: HBeAg-negative CHB was established on HBV DNA > 2000 IU/mL plus fluctuating or persistently elevated ALT levels and/or histological evidence of at least moderate fibrosis and/or necroinflammation; HBeAg-negative chronic infection or IC state was established on persistently normal ALT levels plus HBV DNA < 2000 IU/mL or HBV DNA 2000-20000 IU/mL plus evidence of mild or absent hepatic necroinflammation and fibrosis. Diagnosis of liver cirrhosis was established by liver biopsy (Ishak score 5 or 6) or transient elastography values > 13.1 kPa[12]. This study was conducted in accordance with the Declaration of Helsinki guidelines and the principles of Good Practice and was approved by the Ethics Committee of both hospitals (PR(AG) 245/2015).

Baseline clinical and laboratory assessment

Data on demographics (sex, age and race), toxic exposure (alcohol, tobacco), cardiovascular risk factors (on-treatment arterial hypertension, diabetes and dyslipidemia) and anthropomorphic characteristics (height, weight, and waist circumference) were prospectively collected at the time of enrollment. A blood test was performed including hematology and a standard biochemical panel as well as insulin level, glycated hemoglobin, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, C-reactive protein, HBV serology (quantitative HBsAg, anti-HBc, HBeAg and anti-HBe) and HBV virology. HBV DNA was determined using the COBAS 6800 HBV test (Roche Diagnostics, Mannheim, Germany), with a lower limit of quantification of 20 IU/mL and lower limit of detection of 10 IU/mL. Antibodies against HCV, hepatitis D virus and HIV were also tested.

Central obesity was defined as a waist circumference greater than 102 cm in men and 88 cm in women. Insulin resistance was determined with the homeostasis model assessment[13]. The nonalcoholic fatty liver disease (NAFLD) score was also calculated, and values > 0.675 were considered suggestive of advanced NAFLD-related fibrosis[14]. Liver elastography (Fibroscan® 502 Touch, Echosens, Paris, France), including the control attenuation parameter (CAP) as a marker to quantify hepatic steatosis, was carried out in all patients. CAP was chosen as marker of liver steatosis because it has been pointed out as more accurate than other scores such as Hepatic Steatosis Index in patients with chronic infection by HBV[15].

Carotid artery evaluation

Carotid Doppler ultrasound study (Vivid I, General Electric, GE Healthcare, Horten, Norway, equipped with a 3.5-10 MHz linear transducer) was performed to determine the IMT. B-mode ultrasound with a semi-automatic edge-detection algorithm was used to measure the medium and maximum IMT on the far wall of both the right and left side of the common carotid artery at 1 cm before the bifurcation, measuring at least 250 mm of a straight arterial segment. The presence of an atheroma plaque was established based on the Manheim criteria, as a focal structure that encroached into the arterial lumen by at least 0.5 mm or 50% of the surrounding IMT value or demonstrated a thickness > 1.5 mm as measured from the media-adventitia interface to the intima-lumen interface[16]. The presence of plaques was investigated in the common carotid artery and internal and external carotid arteries. Subclinical atherosclerosis was established on an increased IMT (≥ 1.2 mm) and/or detection of a carotid plaque[16]. To avoid interobserver variability, ultrasound measurements were performed by operators specifically trained in carotid ultrasound cardiovascular risk assessment. Moreover, measurement of the IMT at the common carotid artery presented high reproducibility and interobserver agreement in previous multicenter studies[17].

Statistical analysis

Normally distributed quantitative variables were compared with the Student *t* test and those with a non-normal distribution with the Mann-Whitney *U* test. Quantitative variables were expressed as the median and interquartile range or mean and standard deviation depending on the group size. Categorical variables were compared between groups using the χ^2 or Fisher exact test, as appropriate.Variables with a *P* value < 0.10 in the univariate model were analyzed in a multivariate logistic regression model. Quantitative variables were also introduced as categorical (median or mean of the overall cohort) in order to increase the potency of the models. In the case of homeostasis model assessment, values from included patients were contrasted with the normal from general population[18]. Odds ratios (ORs) and 95% confidence intervals were calculated for the independent predictive factors of carotid plaques and subclinical atherosclerosis. Only patients with available data for all the variables considered in the analysis were included in the multivariate logistic regression models.

Because enrollment of patients with CHB was difficult due to the limitation to naïve subjects, the number of CHB and ICs differed. For this reason, a propensity score analysis matched by sex, age and main cardiovascular risk factors was carried out by using the package of R[19]. *P* values < 0.05 were considered statistically significant. All statistical analyses were performed using IBM SPSS, version 26.0 (SPSS Inc, Armonk, NY, United States).

RESULTS

Baseline characteristics of patients

In total, 402 individuals were enrolled: 201 chronic HBV-infected and 201 healthy controls. Overall, 218 (54.2%) were males, and the more common cardiovascular risk factors were active or former smokers (33.3%), alcohol intake (25.8%) and dyslipidemia (19.9%). Both alcohol intake and central obesity were more common in the control group. Table 1 shows the baseline characteristics of the included cohorts of patients.

In the HBV-infected group, 152 (75.6%) were ICs and 49 (24.4%) CHB. In the latter, 12 (24.4%) patients had liver cirrhosis. Baseline characteristics according to the classification of HBV infection were summarized in Table 1. Most patients were Caucasian (68.2%), and the median age was 47 years. Demographical features did not differ between the two groups. Dyslipidemia was more common in ICs than in patients with CHB, whereas the prevalence of the remaining cardiovascular risk factors was similar. ALT, HBV DNA and HBsAg values as well as liver stiffness were significantly higher

Table 1 Baseline characteristics of included subjects and comparison between infected and non-infected subjects and among patients infected by hepatitis B virus according to the phase of the infection (chronic hepatitis B *vs* inactive carriers)

	Controls	Chronic hepatitis B	Inactive carriers		
	n = 201	*n* = 49	*n* = 152	*P* value[1]	*P* value[2]
Age, yr	48.1 ± 10.2	48.4 ± 12.0	46.5 ± 13.4	0.29	0.28
Male sex (%)	103 (51.2)	31 (64.6)	84 (54.9)	0.13	0.16
Race (%)				< 0.001	0.48
Caucasian	186 (92.5)	35 (72.9)	102 (66.7)		
Asian	2 (1.0)	6 (12.5)	11 (7.2)		
African	0 (0)	6 (12.5)	28 (18.3)		
Hispanic	13 (6.5)	1 (2.1)	12 (7.8)		
Cardiovascular risk factors (%)					
Tobacco exposure	74 (36.8)	15 (31.3)	45 (29.6)	0.09	0.48
Alcohol intake[3]	70 (34.8)	11 (22.9)	23 (15.4)	< 0.001	0.17
Hypertension	40 (19.9)	11 (22.9)	27 (17.8)	0.46	0.28
Diabetes	4 (2.0)	4 (8.3)	6 (3.9)	0.08	0.2
Dyslipidemia	46 (22.9)	3 (6.3)	31 (20.4)	0.08	0.02
Central obesity	52 (25.9)	10 (20.8)	27 (17.9)	0.04	0.4
BMI, kg/m^2	25.3 ± 3.6	26.0 ± 3.9	25.2 ± 4.0	0.76	0.22
Liver cirrhosis (%)	0 (0)	12 (24.4)	0 (0)	< 0.001	< 0.001
ALT, IU/mL	22.6 ± 12.7	59.7 ± 48.6	25.6 ± 16.7	< 0.001	< 0.001
GGT, IU/mL	30.4 ± 30.9	60.7 ± 87.9	31.5 ± 63.3	0.24	< 0.001
LDL, mg/dL[4]	131.9 ± 38.3	116.9 ± 30.7	118.1 ± 32.6	0.002	0.82
Triglycerides, mg/dL	108.0 ± 56.7	96.4 ± 46.3	106.8 ± 59.1	0.54	0.26
C-reactive protein, mg/dL[5]	0.29 ± 0.42	0.84 ± 1.90	1.00 ± 9.00	0.42	0.88
HOMA index[5]	2.05 ± 1.84	4.20 ± 3.50	3.40 ± 3.90	< 0.001	0.18
HBsAg, logIU/mL	-	3.6 ± 0.8	2.9 ± 1.2	-	0.001
HBV DNA, logIU/mL	-	4.4 ± 1.8	2.4 ± 1.1	-	< 0.001
Transient elastography, kPa	4.5 ± 1.4	11.3 ± 10.9	5.5 ± 2.4	< 0.001	< 0.001
CAP, dB/m	246.5 ± 54.5	227.4 ± 55.0	227.2 ± 56.2	0.001	0.98

Data are expressed as the median (interquartile range) or as the *n* (%).
[1]Comparison between hepatitis B virus-infected and non-infected controls.
[2]Comparison between patients with chronic hepatitis B and inactive carriers.
[3]Significant alcohol intake was defined as > 30 g per day for men and > 20 g per day for women.
[4]These data were available in 132 non-infected subjects.
[5]This data was available in 83 non-infected subjects. ALT: Alanine transaminase; CAP: Control attenuation parameter; GGT: Gamma glutamyltransferase; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; BMI: Body mass index; LDL: Low-density lipoprotein; HOMA: Homeostasis model assessment.

in patients with CHB.

Carotid plaques and subclinical atherosclerosis in HBV-infected group in comparison with the control group

No differences were observed between the HBV-infected group and the control group in terms of gender and age, although some cardiovascular risk factors such as central obesity and dyslipidemia were more common among non-HBV infected individuals (Table 1). In fact, although increased values of liver stiffness were observed in patients with HBV infection, CAP levels were higher in subjects within the control group.

Table 2 Factors associated with the presence of carotid plaques and subclinical atherosclerosis

	Subclinical atherosclerosis			Carotid plaques		
	Univariate analysis	Multivariate analysis		Univariate analysis	Multivariate analysis	
		OR (95%CI)	*P* value		OR (95%CI)	*P* value
Age, years	< 0.001			< 0.001		
Age > 50 years	< 0.001	1.45 (1.24-1.48)	< 0.001	< 0.001	1.43 (1.21-1.44)	< 0.001
Male sex	0.004		0.336	0.003		0.212
Central obesity	0.008		0.073	0.007		0.141
Tobacco exposure	0.003		0.081	0.002		0.187
Alcohol intake[1]	0.109		-	0.073		0.929
Arterial hypertension	< 0.001		0.949	< 0.001		0.690
Diabetes mellitus	0.004	1.13 (1.03-1.59)	0.028	0.01		0.082
Dyslipidemia	0.001			< 0.001		0.095
Chronic hepatitis B	0.001	1.23 (1.11-1.41)	< 0.001	0.016	1.18 (1.06-1.34)	0.004
Transient elastography, kPa	0.01		0.090	0.073		0.438
Transient elastography > 5.7 kPa	0.008			0.048		
CAP, dB/m	< 0.001		0.989	< 0.001		0.577
CAP > 238 dB/m	< 0.001			< 0.001		
AST, IU/mL	0.115		-	0.152		-
AST > 27 IU/mL	0.102			0.131		
GGT, IU/mL	< 0.001		0.067	0.001		0.947
GGT > 36 IU/mL	< 0.001			0.001		
Triglycerides, mg/dL	0.011		0.059	0.018		0.957
Triglycerides > 106 mg/dL	0.009			0.009		
LDL, mg/dL	0.651		-	0.180		-
HOMA index	0.038		0.073	0.278		0.105
HOMA index > 1.2	0.150			0.489		
HOMA index > 3	0.002			0.018		

Data are given as mean ± SD or as *n* (%).

[1]Significant alcohol intake was defined as > 30 g per day for male and > 20 g per day for female. At the multivariate logistic regression model only patients with available data for all the variables were included. The cut-off for inclusion was a *P* value < 0.10 in the univariate model. AST: Aspartate transaminase; CAP: Control attenuation parameter; GGT: Gamma glutamyltransferase; CI: Confidence interval; OR: Odds ratio; HOMA: Homeostasis model assessment; LDL: Low-density lipoprotein.

Overall, patients with HBV infection presented higher rates of both carotid plaques (20.9% *vs* 18.4%) and subclinical atherosclerosis (24.4% *vs* 19.4%), though these differences did not reach statistical significance ($P = 0.31$ and $P = 0.14$, respectively). When the three groups were analyzed separately, taking into account the state of HBV infection, we learnt that patients with CHB had higher rates of carotid plaques (32.7%) and subclinical atherosclerosis (40.8%) than controls (18.4% and 19.4%, respectively), as shown in Figure 1. However, the rates were similar when only ICs and controls were compared (carotid plaques: 17.1% *vs* 18.4%, $P = 0.446$; subclinical atherosclerosis: 19.1% *vs* 19.4%, $P = 0.525$). Although the typical cardiovascular risk factors were linked with both carotid plaques and subclinical atherosclerosis on the univariate analysis, on the multivariate (Table 2) the only factors independently associated with the presence of subclinical atherosclerosis were older age (OR 1.45, $P < 0.001$), diagnosis of CHB (OR 1.23, $P < 0.001$) and diabetes (OR 1.13, $P = 0.028$). Similar results were observed

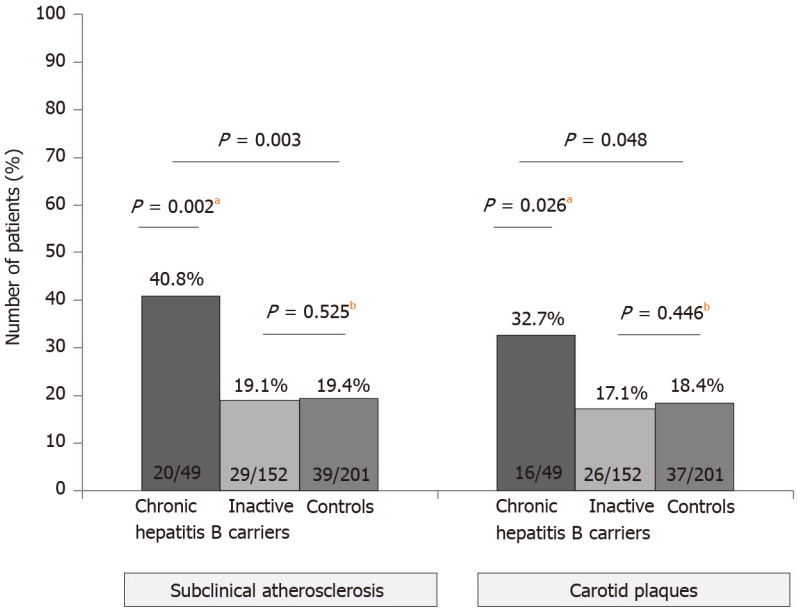


Figure 1 Rate of carotid plaques and subclinical atherosclerosis (defined as intima-media thickness ≥ 1.2 mm and/or presence of atheroma plaques) in the overall cohort. [a]*P* < 0.05; [b]*P* < 0.01.

regarding the carotid plaques, with age over 50 years (OR 1.43, *P* < 0.001) and CHB (OR 1.18, *P* = 0.004) as independent risk factors.

Though this is a prospective study, due to the different number of HBV-infected subjects included in each group, a propensity score analysis including all patients with CHB (*n* = 49) and a cohort with the same number of IC and controls, balanced by age, sex and main cardiovascular risk factors, was carried out. The multivariate analysis of this propensity score revealed similar results as shown the analysis performed with the overall cohort, with older age (OR 1.30, *P* = 0.01) and CHB state (OR 1.26, *P* = 0.03) as independent risk factors associated with the presence of carotid plaques (Table 3).

Carotid plaques and subclinical atherosclerosis in CHB and HBV ICs

Overall, 49 (24.4%) patients had subclinical atherosclerosis, including 42 (20.9%) with carotid plaques, 19 (9.5%) with increased IMT (≥ 1.2 mm) and 12 (6%) with both findings. The prevalence of both subclinical atherosclerosis (*P* = 0.003) and carotid plaques (*P* = 0.019) was higher in patients with CHB than ICs (Figure 2). Liver cirrhosis was associated with an increased risk of subclinical atherosclerosis (42.0% *vs* 23.0%) although the difference did not reach statistical significance (*P* = 0.13). The impact of CHB on the presence of subclinical atherosclerosis remained when patients were stratified by age (Figure 2). In those ≤ 50 years, the prevalence of subclinical atherosclerosis was 12.5% in CHB patients and only 2.2% in ICs (*P* = 0.058). In patients aged over 50 years, those with CHB also had a higher prevalence of subclinical atherosclerosis (68.0% *vs* 45.8%, *P* = 0.051). Age was strongly associated with the presence of subclinical atherosclerosis (*P* < 0.001). On multivariate analysis, factors independently associated with the presence of subclinical atherosclerosis were older age (OR 1.11, *P* < 0.001), increased values of gamma-glutamyltransferase (OR 5.9, *P* = 0.007) and CHB (OR 3.35, *P* = 0.017). When age was introduced as a categorical variable (threshold of 50 years), both CHB and age remained as predictive factors of subclinical atherosclerosis (Table 4).

In terms of carotid plaques, impact of CHB was especially important in patients aged ≤ 50 years (Figure 2). On the multivariate analysis, only age (age > 50 years, OR 1.45, 95%confidence interval 1.30-1.62, *P* < 0.001) and increased gamma-glutamyltransferase levels (gamma-glutamyltransferase > 36 IU/mL, OR 1.19, 95%confidence interval 1.04-1.37, *P* = 0.012) independently impacted the presence of carotid plaques.

Four patients with CHB and liver cirrhosis presented a NAFLD score > 0.675, suggesting significant fibrosis. Two of them had subclinical atherosclerosis, but none had a history of diabetes, and their body mass index was < 25 kg/m^2 and CAP < 250 dB/m. Otherwise, they presented an HBV DNA > 2000 IU/mL, suggesting that fibrosis was likely related to CHB.

Table 3 Factors associated with the presence of carotid plaques in a propensity score matched by age, sex and main cardiovascular risk factors

	Groups of study			Univariate analysis	Multivariate analysis	
	Chronic hepatitis B, *n* = 49	Inactive carriers, *n* = 49	Controls, *n* = 49	*P* value	OR (95%CI)	*P* value
Age, yr	48.4 ± 12.0	48.7 ± 13.0	47.1 ± 11.3	0.78		
Age > 50 yr (%)	25 (51.0)	24 (49.0)	23 (46.9)	0.92	1.30 (1.12-1.50)	0.01
Male sex (%)	31 (64.6)	31 (63.3)	31 (63.3)	1		0.72
Caucasian race (%)	35 (72.9)	32 (65.3)	38 (77.6)	0.41		-
Cardiovascular risk factors (%)						
Tobacco exposure	15 (31.3)	13 (26.5)	16 (32.7)	0.80		0.71
Hypertension	11 (22.9)	10 (20.4)	11 (22.4)	0.96		0.40
Diabetes	4 (8.3)	5 (10.2)	3 (6.1)	0.76		0.57
Dyslipidemia	3 (6.3)	3 (6.1)	5 (10.2)	0.68		0.54
Central obesity	10 (20.8)	10 (20.4)	10 (20.4)	1		0.06
BMI, kg/m^2	26.0 ± 3.9	26.3 ± 4.9	26.4 ± 3.8	0.90		0.16
ALT, IU/mL	48.6 ± 9.4	26.9 ± 9.4	25.2 ± 9.6	< 0.001		0.08
GGT, IU/mL	60.7 ± 87.9	42.7 ± 106.0	37.7 ± 50.9	0.45		0.07
LDL, mg/dL[1]	116.9 ± 30.7	120.3 ± 32.6	112.7 ± 35.3	0.72		0.87
Triglycerides, mg/dL	96.4 ± 46.3	99.0 ± 57.5	115.3 ± 77.1	0.30		0.41
HOMA index[2]	4.2 ± 3.5	4.7 ± 5.8	2.6 ± 2.6	0.14		0.40
Transient elastography, kPa	11.3 ± 10.9	5.7 ± 3.0	5.0 ± 7.9	< 0.001		0.80
CAP, dB/m	227.4 ± 55.0	232.1 ± 48.6	251.5 ± 62.8	0.095		0.49
Chronic hepatitis B state (%)	49 (100)	0 (0)	0 (0)	< 0.001	1.26 (1.09-1.47)	0.03

[1]Only available for 34 controls.

[2]Only available for 25 non-infected controls. ALT: Alanine transaminase; CAP: Control attenuation parameter; GGT: Gamma glutamyltransferase; BMI: Body mass index; LDL: Low-density lipoprotein; CI: Confidence interval; OR: Odds ratio; HOMA: Homeostasis model assessment.

DISCUSSION

The results of this prospective collaborative study including well-characterized HBeAg negative chronic HBV infection show that CHB is independently associated with the presence of both carotid plaques and subclinical atherosclerosis. These results suggest that HBV infection may have a role as a cardiovascular risk factor in naïve patients with CHB.

There are few studies assessing the potential effect of HBV infection on development of carotid atherosclerosis, and they are all cross-sectional with a limited number of HBsAg-positive populations (Supplementary Table 1). In two of these studies, an association was observed between HBsAg positivity and early atherosclerosis[11,20]. The severity of liver disease was not determined in any of these studies, and therefore no data on the possible impact of CHB was reported. In our cohort, similar to HCV and HIV, patients with HBV infection had greater risk of subclinical atherosclerosis and carotid plaques than controls. In this line, a study focusing on early atherosclerosis in liver disease (NAFLD, HCV and HBV) found that all three conditions were strongly associated with early atherosclerosis (OR 1.96, 1.61 and 1.40 respectively), regardless of the patients' classical risk factors, including insulin resistance and metabolic syndrome [20].

Table 4 Baseline characteristics and analyses of factors associated with the presence of subclinical atherosclerosis of patients with hepatitis B virus infection

	Subclinical atherosclerosis	No subclinical atherosclerosis	Univariate analysis	Multivariate analysis		Adjusted multivariate analysis	
	n = 49	*n* = 152	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Age, yr	57 (53.5-62.0)	42 (33.0-52.0)	< 0.001	1.10 (1.06-1.16)	< 0.001	1.19 (1.12-1.25)	< 0.001
Age > 50 yr (%)	45 (91.8)	46 (30.3)	< 0.001	21.9 (6.7-71.8)	< 0.001		
Male sex (%)	31 (63.3)	84 (55.3)	0.207	-	-		
BMI, kg/m^2	70.0 (64-77)	69.5 (62-80)	0.261	-	-		
Central obesity (%)	13 (26.5)	24 (16.0)	0.079	-	0.298		0.356
Tobacco exposure (%)	22 (44.9)	38 (25.2)	0.008	-	0.208		0.920
Alcohol intake[1] (%)	14 (28.5)	20 (13.5)	0.016	-	0.876		0.092
Arterial hypertension (%)	18 (36.7)	20 (13.2)	0.001	-	0.789		0.419
Diabetes mellitus (%)	5 (10.2)	5 (3.3)	0.067	-	0.994		0.457
Dyslipidemia (%)	16 (32.6)	18 (11.9)	0.001	-	0.876		0.786
Chronic hepatitis B (%)	20 (40.8)	28 (18.4)	0.002	3.35 (1.20-9.10)	0.017	1.89 (1.75-2.04)	< 0.001
Liver cirrhosis (%)	5 (10.2)	7 (4.6)	0.138	-	-		
ALT, IU/mL	29 (22.0-43.5)	24 (17.0-34.0)	0.310	-	--		
ALT > ULN (%)	10 (20.4)	22 (14.5)	0.220	-	-		
GGT, IU/mL	31 (18.0-62.5)	20 (16.0-28.0)	< 0.001	-	0.120		
GGT > ULN (%)	16 (32.7)	8 (5.3)	< 0.001	5.90 (1.60-21.40)	0.007	1.27 (1.19-1.36)	< 0.001
HbA1c, %	5.5 (5.3-5.8)	5.4 (5.1-5.6)	0.004	-	0.78		0.551
HbA1c ≥ 6% (%)	7 (14.6)	6 (4.1)	0.018	-	-		
HOMA index	3.3 (2.2-6.0)	2.4 (1.7-3.6)	0.038	-			0.054
HOMA index > 3 (%)	22 (45.8)	51 (34.2)	0.102	-	-		
HBsAg, logIU/mL	3.2 (2.5-3.6)	3.3 (2.4-4.0)	0.321	-	-		
HBV DNA, logIU/mL	3.1(2.4-3.8)	2.9 (2.3-3.5)	0.533	-	-		
Transient elastography, kPa	6.2(4.2-10.3)	5.2 (4.2-6.9)	0.059	--	0.327	1.01 (1.00-1.01)	< 0.001
CAP, dB/m	246 (210.0-289.0)	213 (185.0-261.5)	0.004	--	0.220	1.000 (1.000-1.001)	0.006
CAP > 227 dB/m (%)	29 (67.4)	51 (38.3)	0.001	--	0.172		

Data are given as mean ± SD or as *n* (%).

[1]Significant alcohol intake was defined as > 30 g per day for male and > 20 g per day for female.At the multivariate logistic regression model only patients with available data for all the variables were included (*n* = 250). The cut-off for inclusion was a *P* value < 0.10 in the univariate model. ALT: Alanine transaminase; BMI: Body mass index; HBV: Hepatitis B virus; CI: Confidence interval; CAP: control attenuation parameter; HbA1c: Hemoglobin A1c; GGT: Gamma glutamyltransferase; OR: Odds ratio; ULN: Upper limit of normality; HOMA: Homeostasis model assessment; HBsAg: Hepatitis B surface antigen.

The suggested mechanisms to explain HBV-related atherosclerosis is direct vascular damage by the virus and particularly accelerated oxidative damage and the pro-inflammatory state of chronic HBsAg carriers[21]. Knowledge about the immune response in HBV-infected patients has increased considerably in recent years[9]. The production of proinflammatory cytokines (*e.g.*, interleukin1b, tumor necrosis factoralpha) steadily increases during early life until it reaches the state of chronic low-

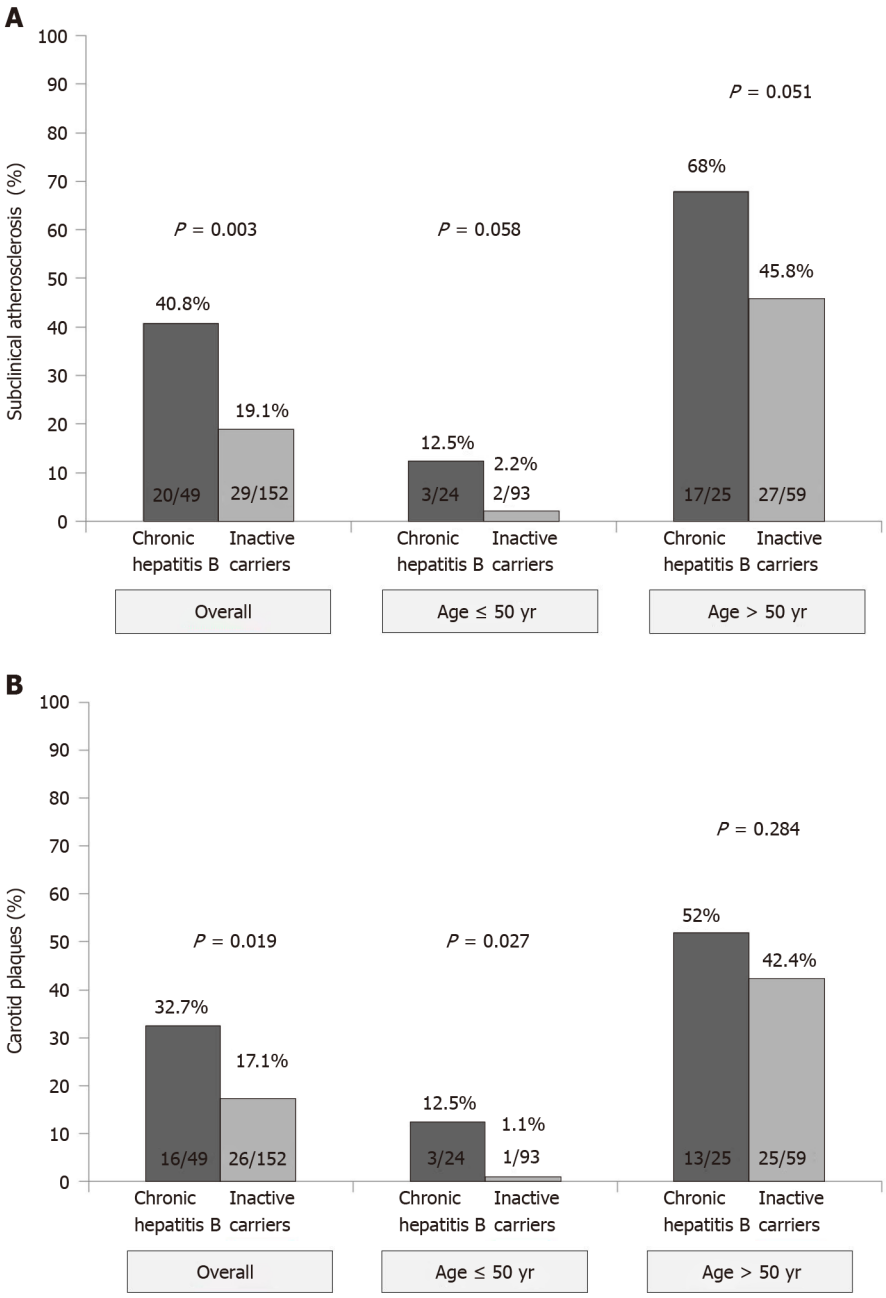


Figure 2 Rate and impact of age and hepatitis B e antigen negative phase of infection (chronic hepatitis *vs* inactive carriers) in subclinical atherosclerosis and carotid plaques in the cohort of patients chronically infected by hepatitis B virus. A: Subclinical atherosclerosis; B: Carotid plaques.

grade systemic inflammation that occurs in elderly persons[22]. HBeAg-negative CHB [3] has been linked with a propensity to mount proinflammatory immune reactions [9]. In this population, liver inflammation is triggered by HBV-specific CD8 T cells, and it is associated with increased levels of chemokines and natural killer cell activation[23]. This proinflammatory state is independent of ALT levels and even HBV DNA levels, which usually fluctuate in this stage of the disease[24]. However, it has been clearly associated with progression of liver disease[9].

In our study, neither ALT levels nor HBV DNA were associated with an increased prevalence of subclinical atherosclerosis. This fact may be explained by the inclusion of patients with CHB with normal ALT but increased values of HBV DNA and liver damage at liver biopsy. On the other hand, some of the patients with liver cirrhosis presented relatively low HBV DNA levels. Older age and CHB status were independent factors associated with increased carotid plaques and subclinical atherosclerosis, in line with the proinflammatory state induced by older age and progression of liver damage.

Serum paraoxonase-1 and arylesterase activities, plasma free sulfhydryl groups and total antioxidant capacity, all factors associated with increased susceptibility to atherogenesis[24,25], are lower in HBV patients than in non-infected controls[26]. This finding can also contribute to the development of atherosclerosis in patients with HBV infection. Moreover, the association between fibrosis progression and exacerbated immune responses in patients with CHB is well established[9,10,27], so this dysfunctional immunological response might also bring an increase in cardiovascular risk.

Accordingly, HCV infection has been linked with increased prevalence of carotid plaques in those patients with evidence of advanced liver fibrosis[4]. In that study, Petta *et al*[4] showed that 73 of 174 HCV patients (42%) had carotid plaques, with older age and liver fibrosis as independent factors associated with carotid atherosclerosis, results in line with our findings because age and CHB were the two variables independently linked with increased risk of both carotid plaques and subclinical atherosclerosis. The role of liver damage is especially relevant in view of the lack of statistical differences when HBV ICs were compared with controls, suggesting that HBV infection may predispose to cardiovascular risk only when it is associated with a proinflammatory state, as described in patients with CHB[9,27].

This study has some limitations. First, the fact that only naïve patients were included turned out in a relatively low number of patients with HBeAg negative CHB and inferior to the cohort of HBV ICs. However, these patients were well characterized, and all met the European Association for the Study of the Liver criteria for CHB, including 24% with liver cirrhosis. Second, there were some differences among the groups. In order to minimize this potential bias, a propensity score was carried out, confirming the role of CHB status as cardiovascular risk factor. Moreover, data presented herein derived from a prospective, collaborative cohort of well-characterized patients, including different ethnicity and therefore HBV genotypes.

Interestingly, since reversion of liver fibrosis in patients with CHB is possible due to nucleos(t)ide analog therapy[28], it would be appealing to assess the potential impact of oral antiviral therapy on early atherosclerosis related to HBV infection, especially to view the effect of antiviral treatment for HCV in the overall cardiovascular risk and specifically in the carotid plaques[7].

CONCLUSION

In conclusion, in this prospective, case-control collaborative study, presence of subclinical atherosclerosis and carotid plaques were more frequent in patients with HBV infection than controls. The presence of liver damage was an independent factor associated with subclinical atherosclerosis and carotid plaques, regardless of the classical cardiovascular factors.

ARTICLE HIGHLIGHTS

Research background

There is an increased risk of atherosclerosis in patients with chronic hepatitis C and also in individuals with human immunodeficiency virus infection.

Research motivation

There is scarce data on the potential role of hepatitis B virus infection as a cardiovascular risk factor.

Research objectives

To assess whether the stage of hepatitis B e antigen (HBeAg)-negative chronic hepatitis B virus infection impacts the presence of both carotid plaques and subclinical atherosclerosis and to evaluate if the risk of both carotid plaques and subclinical atherosclerosis in HBeAg-negative patients differ to those of healthy controls.

Research methods

Prospective case-control study with 402 subjects prospectively recruited at the outpatient clinic. Anthropomorphic and metabolic measures, liver stiffness and carotid Doppler ultrasound were performed.

Research results

Patients with HBeAg-negative chronic hepatitis B presented a higher rate of carotid plaques than healthy controls (32.7% *vs* 18.4%, P = 0.002), but no differences were observed between controls and hepatitis B inactive carriers. HBeAg-negative chronic hepatitis B was an independent risk factor for carotid plaques as well as age, dyslipidemia and central obesity.

Research conclusions

These results suggest that hepatitis B infection may have a role as a cardiovascular risk factor in patients with chronic hepatitis B.

Research perspectives

Further studies should assess the potential impact of oral antiviral therapy on early atherosclerosis related to hepatitis B virus infection.

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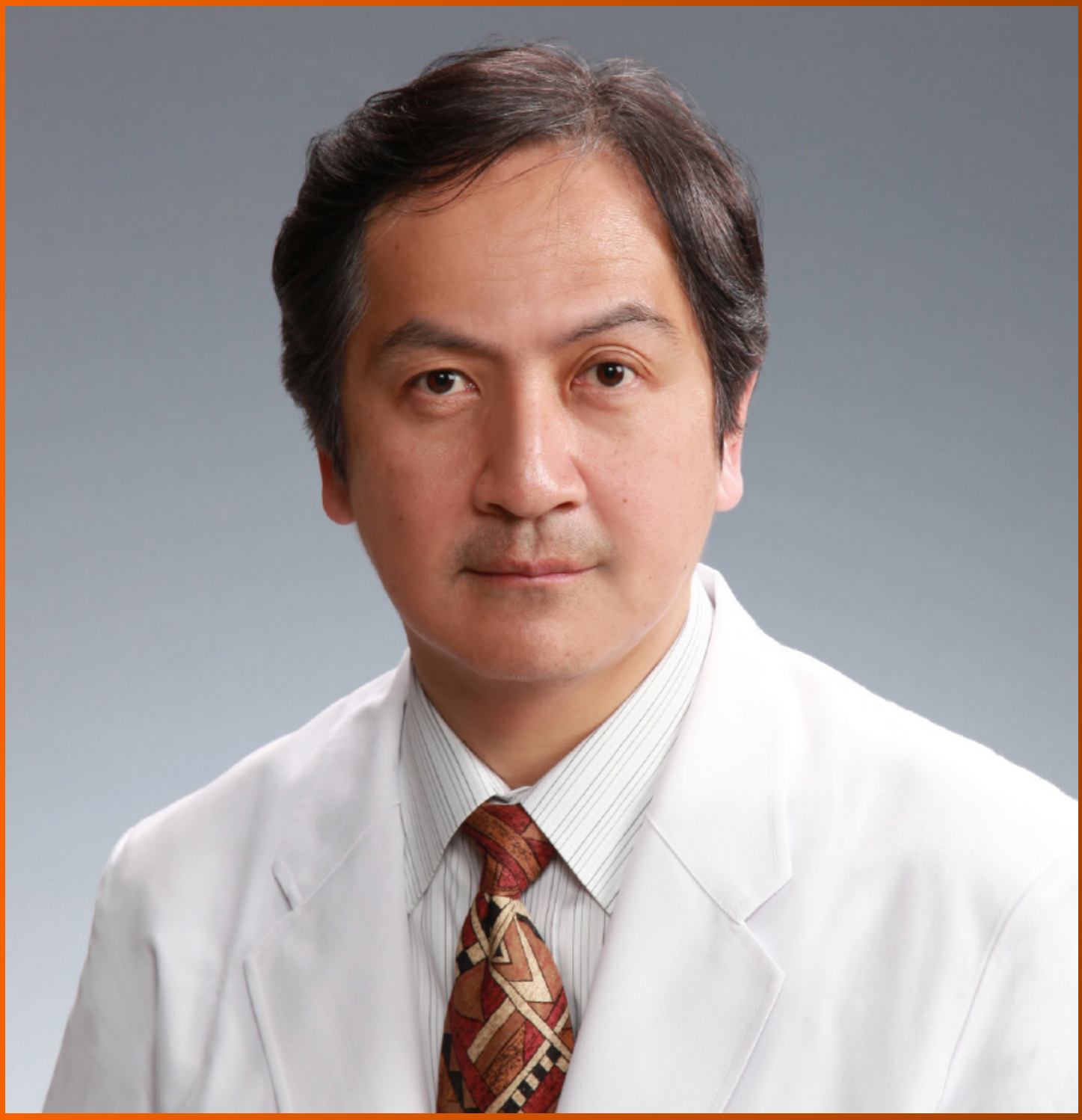


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REVIEW

Endoscopic diagnosis and treatment of gastric dysplasia and early cancer: Current evidence and what the future may hold

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Abstract

Gastric cancer accounts for a significant proportion of worldwide cancer-related morbidity and mortality. The well documented precancerous cascade provides an opportunity for clinicians to detect and treat gastric cancers at an endoscopically curable stage. In high prevalence regions such as Japan and Korea, this has led to the implementation of population screening programs. However, guidelines remain ambiguous in lower prevalence regions. In recent years, there have been many advances in the endoscopic diagnosis and treatment of early gastric cancer and precancerous lesions. More advanced endoscopic imaging has led to improved detection and characterization of gastric lesions as well as superior accuracy for delineation of margins prior to resection. In addition, promising early data on artificial intelligence in gastroscopy suggests a future role for this technology in maximizing the yield of advanced endoscopic imaging. Data on endoscopic resection (ER) are particularly robust in Japan and Korea, with high rates of curative ER and markedly reduced procedural morbidity. However, there is a shortage of data in other regions to support the applicability of protocols from these high prevalence countries. Future advances in endoscopic therapeutics will likely lead to further expansion of the current indications for ER, as both technology and proceduralist expertise continue to grow.

Key Words: Gastric cancer; Endoscopy; Endoscopic imaging; Endoscopic mucosal resection; Endoscopic submucosal dissection

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Core Tip: There have been numerous recent advances in the endoscopic detection, characterization and treatment of precancerous gastric lesions and early gastric cancers. Accumulating evidence suggests that endoscopic submucosal dissection results in at least equivalent disease-related outcomes with a marked reduction in morbidity compared to gastrectomy. Supportive data are robust in regions with high gastric cancer prevalence, however a paucity of western data results in inconsistencies in clinical practice in these regions. This article serves to review existing evidence regarding endoscopic imaging and therapeutics in gastric cancer, as well as identify future areas for research and development.

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INTRODUCTION

Gastric cancer remains a leading cause of cancer-related morbidity and mortality, accounting for 780000 deaths and more than 1 million new diagnoses worldwide in 2020 alone[1,2]. Conventional surgical management with gastrectomy is associated with significant morbidity and mortality[3]. Countries with a high prevalence of gastric cancer have implemented systematic screening programs and demonstrated the benefit of early detection and endoscopic resection (ER) of precancerous gastric lesions and early gastric cancers (EGCs), offering curative treatment with considerably less morbidity[4,5]. Future challenges and opportunities remain, including the judicious surveillance of gastric metaplasia in lower prevalence communities, utilising improvements in endoscopic imaging and further refining existing ER techniques, while at all times accumulating high-quality collaborative data sets to inform and shape future management algorithms.

ENDOSCOPIC DIAGNOSIS OF GASTRIC NEOPLASIA

Endoscopic gastric cancer screening

Rates of gastric cancer in Eastern Asia (particularly Japan and Korea) are markedly higher than Western regions, which has resulted in implementation of population screening programs as an evidence based, cost effective measure for preventing gastric cancer-related mortality and morbidity[4-6]. Screening programs facilitate the detection of precancerous lesions and EGCs at an endoscopically resectable stage. Japanese guidelines suggest biennial or triennial endoscopic screening for those over the age of 50. This approach could prevent up to 63% of gastric cancer related mortality, resulting in 27.2 quality-adjusted life years gained *per* 1000 individuals[4]. In Korea, guidelines similarly suggest biennial screening but commencing at age 40. This is supported by data showing a 38% reduction in age-standardised mortality rate in the screening group at large, at a cost of less than the average gross expenditure product *per* capita[5].

In Western regions including North America and Australasia, the age-standardised incidence of gastric cancer is 6.5-8.8 *per* 100000 population, four-times lower than East Asia[6]. The lower incidence may be attributable to a combination of reduced *Helicobacter pylori* prevalence as well as environmental, lifestyle (diet, smoking) and genetic factors[6]. Given this comparatively low gastric cancer incidence, Western countries are yet to adopt population screening. Instead, guidelines in these regions are varied. The American Gastroenterology Association does not recommend population screening, only surveillance for patients with gastric intestinal metaplasia (GIM), while the British Society of Gastroenterology (BSG) recommends endoscopic screening in patients with multiple risk factors for gastric cancer (male, smoker, pernicious anaemia, family history in first degree relative), citing low-grade evidence[7,8]. Unsurprisingly, low disease prevalence combined with variable guidelines leads to

inconsistencies in clinical practice, as demonstrated in a 2016 survey of American endoscopists[9]. An additional challenge in these low prevalence regions is the management of individuals who have migrated from areas where gastric cancer incidence is high, as data show persistently elevated gastric cancer risk for at least two generations, yet the infrastructure and guidelines in terms of screening are not established[10,11]. These factors would undoubtedly contribute to the discrepancy in gastric cancer survival between Western and East Asian regions, highlighting the need for additional research in these populations[12].

Progression of precancerous gastric lesions

Gastric cancer develops through a well-established precancerous cascade: From atrophic gastritis (AG) to GIM, low-grade dysplasia (LGD), high-grade dysplasia (HGD) and eventually carcinoma, with the likelihood of progression increasing as this cascade advances[13]. Although eradication of *Helicobacter Pylori* has been clearly demonstrated to reduce rates of development of this precancerous cascade, data have been more variable regarding prevention of progression from GIM, implying a 'point of no return' in the gastric cancer cascade[14-16]. These observations underpin the utility of screening and surveillance endoscopy, providing the opportunity to identify and treat curable lesions. In order to maximise yield, guidelines also suggest routine 'mapping' biopsies according to the updated Sydney protocol (including two biopsies taken from the antrum and one from each of the incisura, lesser curve and greater curve) in addition to biopsies of any suspicious areas during surveillance endoscopy, which results in improved AG and GIM detection compared to non-systematic biopsies[8,17].

Japanese data suggests an overall 5-year cumulative gastric cancer incidence of 1.9%-10% in AG and 5.3%-9.8% in GIM[18]. The Operative Link on Gastritis/Intestinal Metaplasia Assessment (OLGIM) scoring system further assists in differentiating between degrees of AG and GIM with regard to gastric cancer risk[19]. Those with high-risk lesions meeting OLGIM stage III/IV have an odds ratio for gastric cancer of 2.41 and 3.99 respectively[20]. Accordingly, guidelines suggest that patients with OLGIM stage III/IV AG or with GIM have 3-yearly surveillance endoscopy or 1- to 2-yearly endoscopy in the presence of a family history of gastric cancer[21]. Although these scoring systems are consistently reported in high-prevalence regions, the limited experience in lower prevalence regions again results in inconsistencies, as pathologists rarely report according to OLGIM staging and thus endoscopist adherence to GIM surveillance guidelines is variable[9].

In the context of biopsy-detected LGD and HGD, the risks of progression to gastric cancer are reportedly between 2.8%-11.5% and 10%-68.8% respectively; therefore guidelines suggest endoscopic removal of any defined lesion with dysplasia[22-26]. Importantly, lesions are also frequently upgraded histologically once endoscopically resected. In a 2015 study, 25% of lesions initially diagnosed as LGD were upgraded after ER; 16.7% to HGD and 6.9% to carcinoma[27]. Accordingly, gastric lesions with any degree of biopsy proven dysplasia should be regarded as potentially housing adenocarcinoma.

Endoscopic imaging techniques

The endoscopic detection and characterisation of EGCs and precancerous lesions is therefore critical in establishing cancer risk and directing appropriate treatment and surveillance. Consensus guidelines from the BSG and the Japanese Gastroenterological Endoscopy Society (JGES) define endoscopic standards to maximise detection of gastric mucosal lesions, including a minimum 7 min gastric examination time, as well as adequate mucosal visualisation using sufficient air insufflation, mucosal cleaning techniques (mucolytic and defoaming agents) and consideration of peristalsis inhibiting medications if views are obstructed by movement[28,29]. While a consensus has not yet been reached regarding the role of advanced endoscopic imaging techniques, a number of recent advances in endoscopic imaging have demonstrated higher detection rates and more accurate characterisation of mucosal lesions.

White light endoscopy: White light endoscopy (WLE) is the conventional endoscopic imaging modality. It is sufficient for detecting some features of carcinomatous transformation of gastric lesions, including red discolouration of the mucosal surface, depressed-type lesions and mucosal ulceration[30,31]. However, the sensitivity for WLE in detecting GIM, LGD/HGD and EGC has been reported to be as low as 29%-59.1%, 51%-74% and 48%-72% respectively[32-36]. As a result, more advanced endoscopic imaging techniques are now used for lesion detection and characterisation.

Chromoendoscopy: Traditional chromoendoscopy requires the topical application of stains or dyes (usually methylene blue, indigo carmine and/or acetic acid) in an effort to enhance tissue characterisation[37,38]. Studies have demonstrated the superiority of chromoendoscopy in the detection of EGCs and precancerous gastric lesions, with a 2016 meta-analysis demonstrating pooled sensitivity of 90% and specificity of 82% [38]. However, the requirement for local dye application has limited its use in the context of the wide field required for gastric lesion detection, particularly following the development of virtual chromoendoscopy.

Narrow-band imaging: Narrow-band imaging (NBI) is the most commonly used form of virtual chromoendoscopy; utilising a rotating optical interference filter to restrict incident light into two narrow bands of different wavelengths (Figure 1). This enhances the definition of the surface mucosa, while emphasising the contrast of the vascular network[39]. NBI is superior to WLE in detection of early precancerous gastric lesions, with pooled sensitivity of 69% and specificity of 91% for GIM[40]. Prospective trials have demonstrated a 40% increase in detection of all focal gastric lesions and more than twice the detection rates for GIM using NBI compared to WLE [32,41].

High-magnification NBI: High-magnification endoscopy (Figure 2) uses a movable lens in the tip of the endoscope to allow up to 150 times optical zoom without any degradation of image quality[42]. This is usually combined with the use of a translucent cap to stabilize the focal length between the lens and the target tissue, as well as near-focus imaging that allows the endoscope to be positioned closer to the mucosal surface[42]. High-magnification endoscopy has been combined with NBI (ME-NBI) to facilitate detailed assessment of the mucosa and enhance lesion detection and characterisation.

Uedo *et al*[42] first described the 'light blue crest' seen on ME-NBI (Figure 2) which has been demonstrated to detect GIM with a sensitivity of 80%-89% and a specificity of 93%-96%[36,43,44]. In regard to characterisation of dysplasia, a prospective multicentre study in 2016 showed an improvement in sensitivity from 74% to 92% with the use of ME-NBI over WLE[36]. Detailed assessment of surface microvascular patterns on polypoid lesions using ME-NBI results in sensitivity of up to 86.2% and specificity of up to 97% for the presence of dysplasia[45].

In the assessment of EGCs, there have been a number of classification systems using ME-NBI. Yao *et al*[45] first described the 'VS' system in 2008, which has a sensitivity of 86%-97% for EGCs[46-49]. This system requires detailed assessment of the microvascular and surface mucosal pattern to ascertain features of irregularity, which in the presence of a demarcation line is suggestive of carcinoma[46]. The VS classification was further simplified by Yamada *et al*[49] in 2014, who demonstrated that the presence of a demarcation line with an irregular microvascular pattern was 95% sensitive and 96% specific for EGC[49].

The accuracy of ME-NBE was confirmed in a 2015 meta-analysis of a combined 2171 patients, demonstrating 86% sensitivity and 96% specificity for the diagnosis of EGC [50,51]. Direct comparator studies of ME-NBI *vs* WLE have also shown up to twice the rate of EGC detection with ME-NBI[52-54].

In addition, ME-NBI is valuable in determining tumour margins at the time of ER, resulting in 97.4%-98.1% accuracy of endoscopic markings[55-57]. Comparator studies have demonstrated the superior precision of ME-NBI *vs* chromoendoscopy in this context, with 17%-20% higher rates of accurate endoscopic markings[55,57]. The use of ME-NBI is also beneficial when lesion margins are unclear on chromoendoscopy, as demonstrated by Nagahama *et al*[57] who found that 72.6% of lesions with an unclear margin on chromoendoscopy were able to be clearly delineated using ME-NBI[57].

Blue laser imaging: Blue laser imaging (BLI) uses laser to create a highly narrowed blue band, enhancing vascular structures without using a filter as is required for NBI, thereby resulting in a higher light intensity[58,59]. There is a paucity of evidence regarding BLI in comparison to other endoscopic imaging, however recent studies have demonstrated superior sensitivity (93%-94%) in detection of EGCs compared to WLE (46%-50%)[60,61]. While direct comparator studies are limited, Kaneko *et al*[61] compared BLI and NBI in 39 patients with gastrointestinal neoplasia and found that BLI maintained sufficient brightness and contrast up to 40 mm while NBI deteriorated beyond 20 mm. This could be of relevance in detection of EGCs where endoscopists may benefit from increased field of view, however further studies in this area are required.

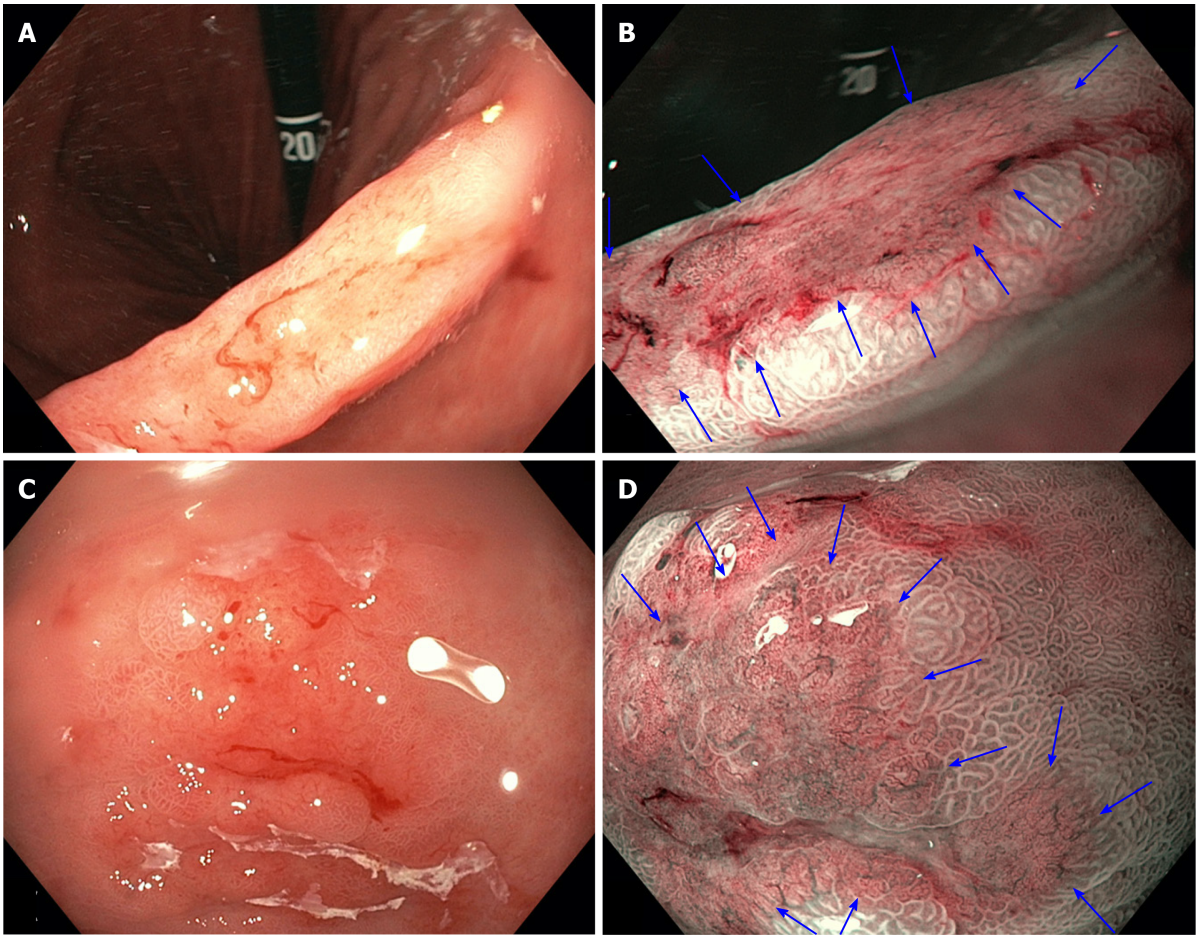


Figure 1 White light endoscopy compared to narrow-band imaging in gastric lesions, demonstrating clear demarcation lines and irregular microvascular/microsurface patterns on narrow-band imaging. A: Early gastric cancer at the incisura seen on white light endoscopy (WLE); B: The same lesion seen on narrow-band imaging (NBI) (blue arrows); C: Early gastric cancer in the antrum seen on WLE; D: The same lesion seen on NBI (blue arrows).

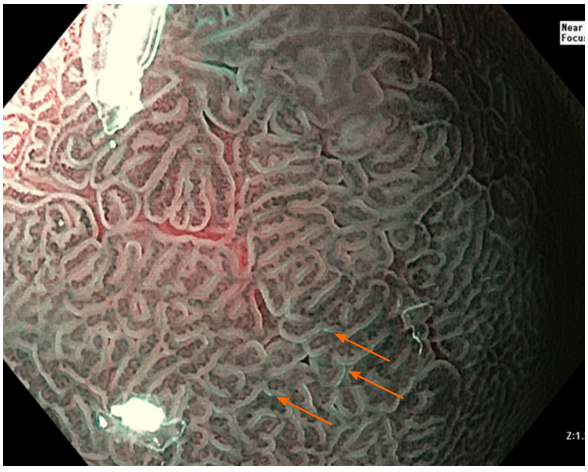


Figure 2 Narrow-band imaging demonstrating the 'light blue crest' (orange arrows) consistent with intestinal metaplasia.

Confocal laser endomicroscopy: Confocal laser endomicroscopy (CLE) uses a low-power laser to illuminate tissue, detecting reflected fluorescent light and allowing high-resolution endoscopic histological assessment[62,63]. CLE was first assessed in precancerous gastric lesions by Guo *et al*[63] who reported sensitivity and specificity of 98% and 95% for the detection of GIM[63]. A more recent meta-analysis demonstrated the accuracy of CLE for the diagnosis of all stages of precancerous gastric lesions and

EGCs, with pooled sensitivity of 92% for GIM, 81% for LGD/HGD and 91% for EGC [64,65]. However, its use is limited by the equipment required (either a dedicated endoscopic system or a probe-based system inserted *via* the therapeutic channel), the time required for image acquisition, as well as the learning curve of image interpretation[63].

Future prospects

Artificial intelligence (AI) has demonstrated efficacy in detection and classification of multiple gastrointestinal lesions[66]. Real-time AI gastric lesion detection has not yet been studied, however convolutional neural networks have been generated from endoscopic images with a sensitivity of 92% using WLE and 97% using ME-NBI[67-69]. This could be of particular benefit for improving lesion detection and characterisation for endoscopists without expertise in advanced imaging interpretation.

Texture and colour enhancement imaging (TXI) is a recently developed technology aiming to improve lesion detection by enhancing texture, brightness and colour tone (Figure 3) using stacked images, while maintaining a similar colour spectrum to WLE [70]. By improving lesion detection within WLE, TXI may facilitate superior detection rates during endoscopic screening, although studies are required to investigate this.

Whilst WLE remains the mainstay for the majority of endoscopists, accumulating evidence as presented above points to a significant additive benefit in repeat interrogation of suspicious areas with chromoendoscopy. The trade-off between time, cost, availability and feasibility means that most proceduralists employ virtual chromoendoscopy with ME-NBI.

Assessment of invasion depth and nodal metastases

Endoscopic ultrasound: While computed tomography (CT) and positron emission tomography/CT are endorsed by guidelines for the assessment of distant metastases and locally advanced gastric cancers, endoscopic ultrasound (EUS) has been recommended for EGCs, in particular for distinction of T1 and T2 lesions[71]. However, data on the utility of EUS in assessment of EGC depth of invasion have been varied. Han *et al*[72] reported under-staging in 16.7% of T2 gastric cancers with EUS, and Choi *et al*[71] reported no advantage of EUS over conventional endoscopic assessment using surface nodularity and fold convergence[71]. In comparison, Mouri *et al*[73] reported that lesions classified by EUS as being intramucosal or submucosal border lesions were histologically either intramucosal or invading < 500 μm into the submucosal space (SM1) in 99% and 87% of cases[73]. A 2015 Cochrane review also demonstrated 87% sensitivity for submucosal invasion[74,75]. Regarding specific EUS characteristics, Kim *et al*[76] reported that an arch-shaped submucosal deformity on EUS was associated with a negative predictive value for SM2 (> 500 μm submucosal invasion) or greater of 94.4%, with a sensitivity and specificity of 84% and 83%[76]. More recently, EUS miniature probes have been demonstrated to have higher resolution but less penetration compared to conventional EUS[77]. Miniature probes have demonstrated superiority for assessment of submucosal invasion, with an overall accuracy of 84.5% *vs* 61.6% using conventional EUS when assessing lesions less than 2 cm in diameter without endoscopic features of submucosal invasion[78]. While the role of conventional EUS in the confirmation of suitability for ER remains unclear, miniature probe EUS may have a role in < 2 cm lesions which are indeterminate endoscopically.

Concurrent to these improvements in EUS, the addition of technologies such as CT with multiplanar reformations and virtual gastroscopy has led to overall accuracy as high as 94% for the diagnosis of EGC[79]. In addition, high-speed magnetic resonance imaging (MRI) and diffusion-weighted imaging have also addressed many of the limitations of MRI for the assessment of T-stage, though this remains infrequently used in the assessment of EGCs[80,81]. While a detailed comparison of radiological methods for staging gastric cancers is beyond the scope of this review, improvements in these technologies may facilitate accurate non-invasive assessment of EGCs in the future.

With respect to exclusion of nodal disease prior to ER for EGC, the use of EUS has again been associated with variable accuracy. In a 2008 meta-analysis, the pooled sensitivity of EUS for N1 disease was as low as 58.2%[82]. More recent data have shown an improved sensitivity and specificity of 74%-83% and 67%-70% in detecting nodal positivity[75,83,84]. However, this improved accuracy still remains inadequate to guide consideration of endoscopic management of disease with metastatic potential. Despite technological advances, CT also remains inaccurate for the detection of nodal metastases, with sensitivity between 62.5%-91.9%[85]. Accordingly, gastric cancers

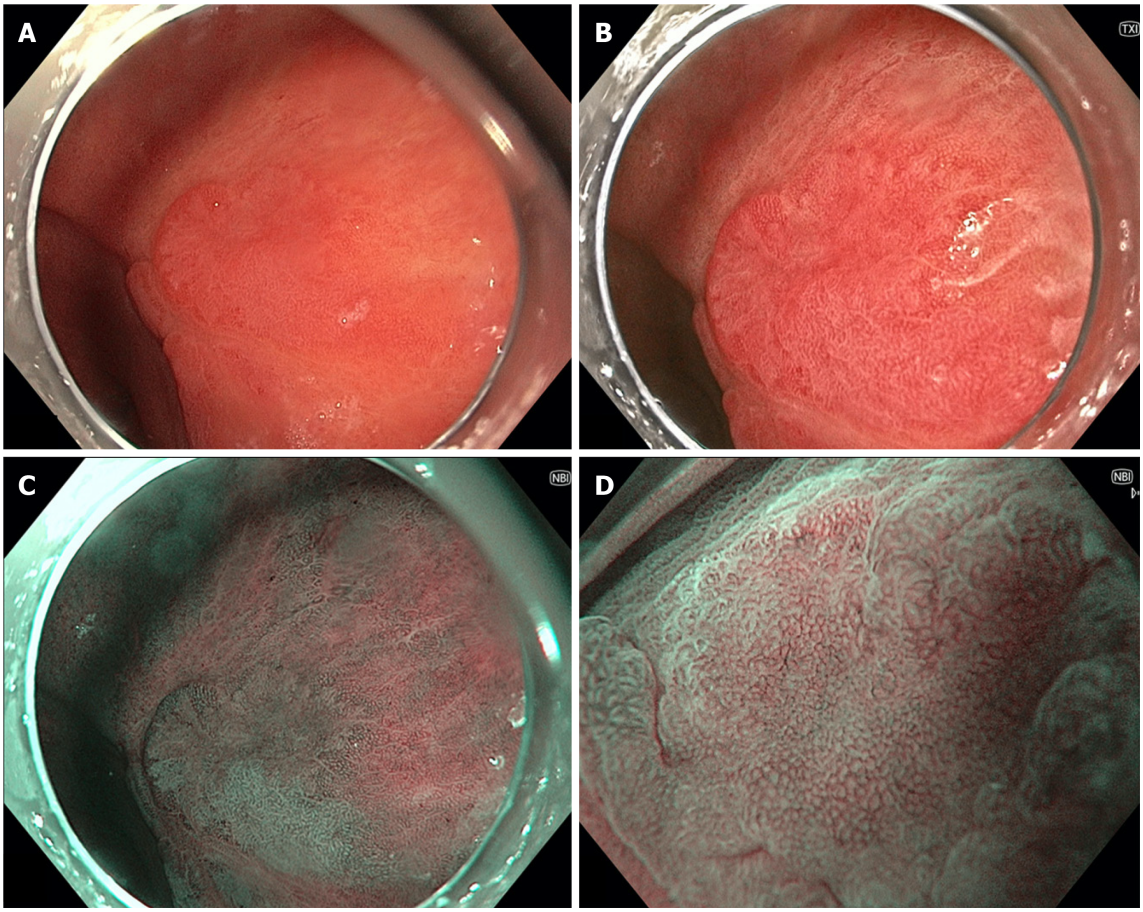


Figure 3 Gastric body lesion with low-grade dysplasia seen on multiple forms of endoscopic imaging. A: White light endoscopy; B: Texture and colour enhancement imaging; C: Narrow-band imaging (NBI); D: high-magnification NBI.

with a significant potential for lymph node metastasis based on lesion characteristics are generally managed surgically with lymphadenectomy to evaluate for lymphatic involvement, as reliable non-invasive exclusion of lymph node metastases (LNM) remains elusive.

ENDOSCOPIC TREATMENT OF GASTRIC NEOPLASIA

ER is indicated for all discrete gastric lesions with histological evidence of dysplasia given the risk of concurrent or future EGC. In addition, data supports ER for lesions histologically indefinite for dysplasia, as the rate of true dysplasia or EGC in resected specimens is as high as 90.8%, particularly in males; lesions > 5 mm in diameter; or lesions with erosions[86]. For gastric mucosal lesions < 1 cm in diameter with LGD, either endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) can be performed, however ESD is preferred in larger lesions and those demonstrating HGD/EGC[87].

Indications for ESD

In the context of histologically confirmed carcinoma, ESD was initially indicated only for the resection of macroscopically intramucosal (T1a) differentiated gastric carcinomas < 2 cm in diameter without ulcer or scar[87]. These have subsequently been labelled 'absolute criteria', as data now support the efficacy of ESD for the resection of a broader range of EGCs termed 'expanded criteria'. In the 2015 JGES guidelines, expanded criteria lesions include all differentiated T1a EGCs > 2 cm without ulcer/scar, differentiated T1a lesions < 3 cm with ulcer/scar, and undifferentiated EGCs < 2 cm in diameter, while the 2015 European Society of Gastrointestinal Endoscopy (ESGE) guidelines also include differentiated lesions < 3 cm with superficial submucosal invasion (SM1 ≤ 500 μm)[87,88].

Another evolving potential indication for ESD is for the resection of submucosal gastric tumours. He *et al*[88] first demonstrated the efficacy and safety of ESD for gastrointestinal stromal tumours (GIST) in 2013, when they reported successful ESD in 25 large gastric GISTs with a mean diameter of 2.7 cm[88]. More recently, An *et al*[89] published data on 168 cases of ESD for gastric GISTs, with complete and en bloc resection in 100% of lesions[89]. Delayed bleeding occurred in only 1.2% and there were no other significant complications. While additional data are required before recommending ESD as a standard of care option for GIST management, early data support this as a potential future indication.

Efficacy of ESD

Data on the efficacy of ESD (Table 1) are particularly robust in East Asian countries where the incidence of gastric cancer is significantly higher than it is in Western countries. Studies in Japan and South Korea have demonstrated en bloc resection in 95.3%-99.2%, complete resection in 87.7%-95.5% and curative resection in 81.7%-84.1% [90-94]. In Western countries however, data are more limited and results are variable. En bloc resection rates are between 92%-97.8%, with complete resection in 75.6%-89% and curative resection in 72.2%-79.2%[95-100].

One contributing factor to this discrepancy is the differing incidence of gastric cancer, resulting in comparatively limited experience in Western centres. This was supported by a 2020 Italian survey where only 41% of included interventionalists had performed more than 80 ESDs and 31% had performed < 40[98]. This study also demonstrated that rates of en bloc resection were higher and rates of perforation lower in more experienced proceduralists. However, there are multiple other contributing factors. Firstly, there are differences in lesion classification, as the Japanese classification of gastric carcinoma includes EGCs which would by European guidelines be classified as HGD[101]. The non-aggressive and non-invasive nature of HGD would result in bias in favour of Japanese outcomes. Further to this, there appear to be differences in disease behaviour in East Asian compared to Western populations. Studies in Korea reviewing rates of LNM in surgical specimens at gastrectomy have reported LNM in 0.3% of absolute criteria lesions and 0.4% in expanded criteria, while marginally higher at 3.2% in undifferentiated carcinomas[102-104]. In contrast, American studies report LNM in 7.5%-13.6% of expanded criteria lesions at gastrectomy[105,106]. Guidelines developed based on data from East Asian populations should therefore be used with caution in Western populations until further evidence is able to clarify this.

EMR *vs* ESD: ESD is associated with longer procedure times and an almost three times higher perforation rate compared to EMR, however multiple meta-analyses have established the superiority of ESD over EMR in regard to en bloc resection rates (OR 9-10 for ESD), complete resection rates (OR 5.7-8.4) and curative resection rates (OR 2.9 [38,107,108]). Tao *et al*[107] also reported a reduction in local recurrence (OR 0.18), likely resulting from the aforementioned superior rates of en bloc, complete and curative resection[107].

ESD *vs* surgery: Increasing evidence suggests that ESD is associated with equivalent overall survival compared to gastrectomy, while reducing procedural complications. A 2015 retrospective Chinese study used propensity score matching to compare outcomes for surgery and ESD in 176 patients with a median follow up of 77 mo[109]. There was no significant difference in overall survival, and while local recurrence occurred in 1.7% of ESD, all but one of the recurrent lesions were treated endoscopically. There was no difference in early complication rates, but late complications were significantly more common in the surgical group (6.8% *vs* 0%). A similar 2017 Korean study found no difference in 5-year survival between groups, with a complication rate of 15% in gastrectomy *vs* 5.1% in ESD[110]. In regard to expanded criteria lesions, data have also reported equivalent survival with a reduction in complications using ESD, with a 2017 study actually demonstrating improved 5-year survival after propensity score matching in the ESD group (97.1% *vs* 85.8%)[109,111,112]. Unsurprisingly, a recent meta-analysis reported higher rates of local recurrence and metachronous cancer in the ESD group, resulting in lower disease-free survival (HR 4.58)[113]. However, the majority of recurrence is able to be treated with repeat ESD and thus lower disease-free survival is not reflected in any difference in disease-specific survival [109,113]. Further to this, the same meta-analysis reported a mean 128 min shorter operation time, 7 d shorter hospital stay, lower procedure-related death and fewer complications[113].

Table 1 Studies reporting *en bloc*, complete and curative resection rates using endoscopic submucosal dissection

Ref.	Location	Total lesions	*En bloc* resection	Complete resection	Curative resection
Suzuki *et al*[90], 2019	Japan	10821	99.2%	91.6%	81.7%
Ryu *et al*[91], 2018	Korea	1541	97.3%	95.5%	N/A
Chung *et al*[92], 2009	Korea	1000	95.3%	87.7%	N/A
Watanabe *et al*[93], 2017	Japan	511	97.4%	92.9%	84.1%
Ngamruengphong *et al*[94], 2020	North America	347	92%	82%	N/A
Manta *et al*[95], 2020	Italy	299	97.6%	89%	72.5%
Abdelrahim *et al*[96], 2019	Europe	175	92.5%	83.4%	N/A
Tate *et al*[99], 2019	Australia	135	94.8%	86.7%	79.2%
Pagano *et al*[98], 2019	Italy	41	97.8%	75.6%	72.2%

N/A: No application.

In regard to complications of surgical management, gastrectomy is associated with significant post-operative complication rates of 9.6%-18.1% for laparoscopic and 17.4%-29.3% for open gastrectomy, as well as a 30-d mortality rate of 2%-4.1%[3,114-116]. In addition, Yu *et al*[116] assessed quality of life scores after gastrectomy and found that fatigue, diarrhoea, reflux, dysphagia and eating restriction scores all remained persistently worse at 5-year follow-up after surgery[116].

A 2015 study demonstrated the lower overall medical costs of ESD compared to surgery for EGC, with ESD costing a median $2374 USD compared to $4954 USD for surgery ($P < 0.001$)[117,118]. This was confirmed by Shin *et al*[109] who reported approximate total hospital stay costs averaging $1871 USD for ESD *vs* $5925 USD for subtotal gastrectomy and $6476 for total gastrectomy[109].

Long-term outcomes: Long-term data on ESD for EGCs has been extensively reported in Japan and South Korea, where studies including up to 5-9 years of follow-up have documented local recurrence rates of 0%-1.8% in absolute criteria lesions and 0.6%-7.0% in expanded criteria lesions[119-123]. In these same cohorts there were no cases of LNM in absolute criteria lesions, and LNM rates were between 0%-0.48% in expanded criteria lesions. Min *et al*[123] reported long-term outcomes (48 mo follow-up) for only lesions meeting criteria for curative resection, in whom 0.3% of absolute and no expanded criteria lesions had local recurrence[123]. In a study by Suzuki *et al*[124] in 2015 (again reporting only on curative resections), despite overall 5-year overall survival being as low as 92.2% due to other comorbidities, the 5-year disease-specific survival was 99.9% in both absolute and expanded criteria lesions[124]. In Western populations, long-term data are limited, but studies have reported local recurrence rates of 4.8%-7% for expanded criteria lesions[125-127].

ESD in 'outside of criteria' lesions: ESD is often also employed for the treatment of lesions outside of even the expanded criteria when patients are unsuitable for major surgery. Abe *et al*[127] followed 14 patients who had ESD for undifferentiated non-curatively resected lesions who were not suitable for or had declined further surgery [127]. Over a median of 76.4 mo follow-up, there was only one case of local recurrence which was managed with repeat ESD. A 2013 Japanese study reviewed 104 patients who undergone ESD despite being outside of criteria in regard to lesion characteristics, who had declined or been deemed unsuitable for surgical management[128,129]. Patients were followed-up for a median of 47 mo after resection, with 5-year overall survival of 70% but disease-specific survival of 91.5%. In patients with comorbidities precluding them from major surgery, ESD may therefore be beneficial even in lesions outside of the expanded criteria.

Approach to incomplete and non-curative resection

Incomplete resection: The limited available evidence supports repeat ESD where possible for lesions with positive horizontal margins after initial resection. Jung *et al*[129] reported 28 patients with a positive horizontal margin after initial ESD, in whom the curative resection rate of repeat ESD was 89.3%[129]. In a 2018 study, Jeon *et al*[130] also reported improved disease-free survival following repeat ESD for non-

curative resections due to positive lateral margins (89.2% *vs* 69.1%)[130].

Non-curative resection: The management of lesions determined to be beyond the expanded criteria histologically after ER (non-curative resection) remains controversial. A large 2019 meta-analysis demonstrated improved 5-year overall and disease-specific survival (OR 3.5 and 3.99 respectively) following additional surgery after non-curative resection[131,132]. However, these data are retrospective and are therefore susceptible to bias associated with the selection of less systemically unwell and comorbid patients in the additional surgery group. Other studies have reported rates of LNM or residual tumour as low as 5.1%, with differences in overall survival but no difference in disease-specific survival between those treated with additional gastrectomy and those managed with surveillance[133,134]. These studies have hypothesised that further data may delineate a group in whom surveillance after non-curative resection is associated with equivalent long-term outcomes.

Multiple studies have addressed factors to stratify the risk of LNM or residual tumour in patients who have had non-curative ESD. In regard to LNM, lymphovascular invasion (LVI) and depth of submucosal invasion histologically are reported to be the most consistent predictors[135,136]. Goto *et al*[134] reported that the presence of depth of invasion > SM1 or LVI was 100% sensitive and 86% specific for the presence of LNM[135]. For predicting the presence of residual tumour, a 2020 meta-analysis reported significant risk factors to be tumour size > 30 mm and the presence of positive horizontal margins[137]. Although additional data are required to guide decision making, patients who have non-curative resections may not warrant additional surgery provided the invasion depth is ≤ SM1 without evidence of LVI, while patients with tumour size > 30 mm or positive horizontal margin may be appropriate for repeat ESD.

This concept led to a 2017 study by Hatta *et al*[137], who developed the eCura scoring system for predicting the risk of LNM using data from 1101 patients who underwent radical surgery after non-curative ESD[137]. The presence of a positive vertical margin, submucosal invasion > SM1, tumour size > 30 mm and vascular invasion were each assigned 1 point, while lymphatic invasion was assigned 3 points. Patients scoring 0-1 points were classified as low-risk (2.5% risk of LNM), 2-4 points intermediate-risk (6.7% risk of LNM) and 5-7 points high-risk (22.7% risk of LNM). The scoring system was then validated on 905 patients with non-curative ESD who declined surgery. 5-year cancer-specific survival was 99.6% in the low-risk group (60.4% of patients), 96% in the intermediate risk group (27.6% of patients) and 90% in the high-risk group (11.9% of patients), suggesting that ESD without additional surgical management may be sufficient in low-risk patients according to the eCura system[138].

These risk stratification tools are of particular importance when considering patients with radiologically enlarged lymph nodes that may or may not signify the presence of LNM. Lee *et al*[138] reported 47 cases of ESD for expanded criteria lesions, where patients had at least one enlarged lymph node on CT[138]. 12 of 47 patients had surgical resection, with no evidence of metastatic disease, while the remaining 35 patients had serial CT monitoring over a median 56 mo. Of these 35 patients, 21 patients had reduction in size or complete resolution of lymphadenopathy, while 13 patients had no change. Only one case had progressive lymph node enlargement but declined biopsy due to age. Assessment of the risk of LNM may therefore help direct decision making regarding the need for additional surgery in the context of radiologically enlarged lymph nodes.

In patients at risk of LNM, future studies may explore the role of ER with additional laparoscopic lymph node resection. A 2011 study by Cho *et al*[139] described 9 patients who had endoscopic full thickness resection (FTR) with laparoscopic regional lymph node resection[139]. Despite 4 lesions having histological LVI, there were no cases of LNM. There may also be a future role for targeted lymph node resection using sentinel node detection strategies. While data have been inconsistent regarding the accuracy of gastric sentinel node detection, these strategies have been most successful in T1 EGCs and thus may be of use following higher-risk ESD[140,141]. Studies using dye have reported a sensitivity of only 75%, however in the context of dual tracer with radiolabelled tin colloid and blue dye, the sensitivity for LNM is up to 93%, with an overall accuracy of 99%[142]. Further studies are therefore required to assess the adequacy of sentinel node guided laparoscopic lymph node resection as a treatment strategy after non-curative ESD.

Complications of ESD

While ESD offers the promise of reduced morbidity, both acute (bleeding, perforation)

and chronic (stenosis, recurrence, metachronous lesions) complications can occur[107,108,113].

Bleeding: The risk of delayed bleeding with ESD is equivalent to that in EMR, with most studies reporting rates between 4% and 6%[143-146]. Nam *et al*[145] retrospectively reviewed 1,864 cases of ESD, in which post-procedural bleeding occurred in 4.1% of patients, with the majority occurring within 24 h of ESD[145]. In their multivariate analysis, predictors of delayed bleeding were patient age ≤ 65 years, resection size > 30 mm, procedure time > 20 min, lesions located in the lower third of the stomach and the presence of erosions. In regard to treatment, delayed bleeding after ESD is generally amenable to routine endoscopic management[146].

Studies have addressed prevention strategies for post-ESD bleeding, with data supporting post-operative proton-pump inhibitor (PPI) use after ESD with an OR of 0.4-0.49 for delayed bleeding[147,148]. A 2016 meta-analysis on the use of PPI prior to ESD found a reduction in gastric pH at the time of procedure, but no pooled difference in delayed bleeding[149]. Previously, routine re-look endoscopies were performed in many centres after ESD in an attempt to prevent rebleeding; a method extrapolated from data supporting second endoscopy after treatment of bleeding peptic ulcers [150]. However, Goto *et al*[144] reported no difference in rates of bleeding before or after routine follow-up endoscopy within one week of initial ESD, suggesting limited benefit from this strategy[144].

Perforation: Perforation occurs more frequently in ESD than in EMR[107,108]. Rates of perforation are generally reported between 1.5% and 9.6%, with a large meta-analysis by Arezzo *et al*[150] demonstrating a pooled perforation rate of 4.9%[95,100,151-156]. Risk factors for perforation include lesions located in the upper third of the stomach, the presence of submucosal invasion or fibrosis and longer procedure times[152,153,157]. Longer procedure times may reflect either more complicated resections or less proceduralist experience, while perforations in upper third gastric lesions likely reflect thinner proximal gastric wall in comparison to the antrum[158].

When macroscopic perforations occur during ESD, more than 97% of cases are able to be treated endoscopically with clip closure[155,156]. Micro-perforations, or suspected perforations that are not endoscopically visible, are usually able to be conservatively managed with fasting and intravenous antibiotics[155].

Delayed perforation, when no intraprocedural perforation occurs but symptoms of peritonism and radiological evidence of perforation develop post-procedure, is a phenomenon that appears to be specific to ESD. It is most common in the thin upper third of the stomach and the majority of cases occur within 24 h[159,160]. Yamamoto *et al*[158] reported 5 cases of delayed perforation in a 2017 case series, of which 4 out of 5 were able to be managed endoscopically[158].

Stenosis: Post-ESD stenosis occurs most commonly following resection of gastric antral (occurring in up to 7% of cases) or cardiac lesions, presenting with either oesophageal or gastric outlet obstruction[161,162]. Sumiyoshi *et al*[160] analysed predisposing factors, with the only risk factor after multivariate analysis being a > 75% circumferential post-resection defect[160]. In regard to treatment, while data is limited, most patients have improvement in their symptoms following either balloon dilation if severe, or conservative management if mild-moderate symptoms[162,163].

Local recurrence and metachronous gastric lesions: Guidelines recommend close ongoing surveillance after ESD for EGC, with ESGE and JGES guidelines suggesting 3- to 6-monthly and 6- to 12-monthly surveillance endoscopy respectively[87,88]. The aim of surveillance after ESD is for early detection of not only local recurrence but also metachronous precancerous gastric lesions. These lesions occur in up to 6.9%-13% of patients after ESD, with a 2019 study reporting a cumulative incidence of metachronous gastric cancer of 33.2 cases *per* 1000 patient-years[95,164,165]. In regard to treatment of metachronous lesions, Kim *et al*[165] reported 117 cases of metachronous gastric neoplasms, of which 77% were able to be retreated with curative ESD[165].

FUTURE DIRECTIONS FOR THE ENDOSCOPIC TREATMENT OF GASTRIC NEOPLASIA

Methods for generating counter-traction

Generating counter-traction during ESD is of benefit in reducing procedural time and

complications[166,167]. Depending on the position of the lesion being resected, gravity may not provide sufficient access to submucosal tissue planes. There have been a number of recent developments with regard to endoscopic equipment and techniques to generate counter-traction.

Magnetic anchor guided ESD: Magnetic anchor guided ESD uses a large external electromagnet combined with an internal magnet attached to the lesion *via* a clip (Figure 4). No comparative studies have assessed the advantages of this technique over conventional ESD, however studies have shown feasibility as well as subjective benefit from the point of view of endoscopists[168,169]. The main limitations of this technique are the equipment required and the coupling strength of the magnets relative to the abdominal wall thickness[170].

Dual channel endoscope: This technique requires a dedicated dual working channel endoscope, often employed for the 'grasp-and-snare' technique in EMR[171]. Hua *et al* [171] compared conventional ESD (24 patients) with dual channel ESD (22 patients), using one channel for dissection while grasping the lesion *via* the second channel[171]. Mean procedure times were significantly shorter with dual channel ESD (20.5 min *vs* 49.1 min) with no other differences in outcomes or complications. There are two main limitations of dual channel endoscopy: The requirement for a dedicated endoscope and the limited distance between working channels inhibiting angulation of traction.

Additional working channel: The use of an attachable additional working channel (Figure 5) aims to overcome some of these limitations of dual channel endoscopy by providing greater distance between working channels as well as allowing the use of a conventional therapeutic endoscope. The additional working channel consists of a flexible attachment, a long shaft and an adaptor for fixation at the endoscope handle [172,173]. This technique has been compared with conventional ESD in porcine stomachs, where it resulted in a reduction in procedure time (24.5 min *vs* 32.5 min) and lower rates of muscularis damage (3.13% *vs* 18.75%)[173]. However, minimal data exist in humans apart from feasibility studies reporting successful resection of 8 lesions by ESD and 6 lesions by EMR[174,175]. The main limitation of this method is the inability to generate significant angulation on the grasping forceps, preventing the most effective direction of counter-traction.

Double endoscopes: Using two separate endoscopes allows maximal angulation for the grasping forceps, optimising counter-traction. Generally, the first endoscope is inserted and a circumferential incision is made. Following this, a second smaller calibre endoscope is inserted to grasp and lift the edge of the lesion while the proceduralist proceeds with dissection. In 2017, Ogata *et al*[175] demonstrated the efficacy of this method in 122 patients, with a 97.5% en bloc resection rate and 86.9% curative resection rate, with perforation and delayed bleeding in 3.3% and 2.5% respectively [175]. While there are no direct comparator trials with conventional ESD, Çolak *et al* [176] employed this method in 6 patients where positioning made conventional ESD difficult[176]. They reported no difference in resection times compared to more straightforward ESDs. There are a number of obvious limitations to this method, including the requirement for two endoscopists, endoscopes and light sources, as well as space limitations in both the oral cavity and within the lumen where one endoscope can obstruct the view of the other[176]. Other groups have reported various methods to overcome some of these limitations. Higuchi *et al*[177] reported double endoscope ESD using a single light source, where the light source from the first endoscope is removed and attached to the second endoscope for insertion until the lesion is grasped [177]. Following this, the light source is reattached to the initial main endoscope. This technique resulted in improved accuracy of dissection compared to historical controls, without any serious intraprocedural complications. Alternatively, Ahn *et al*[178] employed a trans-nasal endoscope for applying traction, maximising space within the oral cavity and within the gastrointestinal tract due to the smaller calibre endoscope [178].

Endo-lifter: The 'endo-lifter' (Figure 6) consists of a transparent hood with grasping forceps, mounted on the tip of an endoscope, with the forceps running externally to the endoscope to allow dissection *via* the working channel[179]. The attachment of the forceps to the hood produces an arc that aims to provide superior angulation for countertraction. Minimal data exist using the endo-lifter in animal models, with variable results. Schölvinck *et al*[180] reported shortened procedure times when used by ESD-experienced but not inexperienced endoscopists[180]. Teo *et al*[179] reported increased visualisation of the submucosa and a reduction in subjective procedural

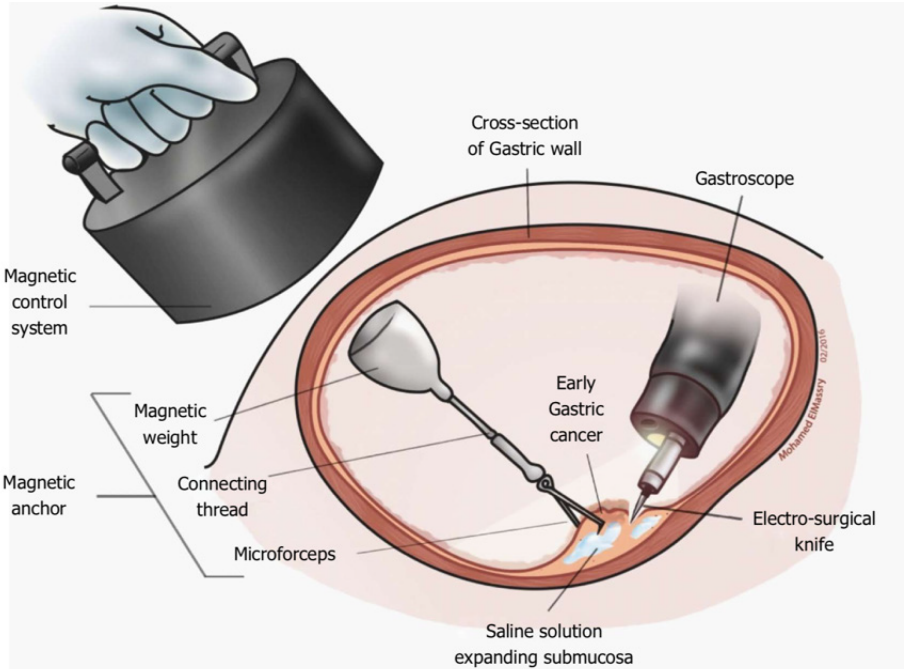


Figure 4 Magnetic anchor-guided endoscopic submucosal dissection[169]. Citation: Mortagy M, Mehta N, Parsi MA, Abe S, Stevens T, Vargo JJ, Saito Y, Bhatt A. Magnetic anchor guidance for endoscopic submucosal dissection and other endoscopic procedures. *World J Gastroenterol* 2017; 23: 2883-2890. ©The Author(s) 2017. Published by Baishideng Publishing Group Inc.

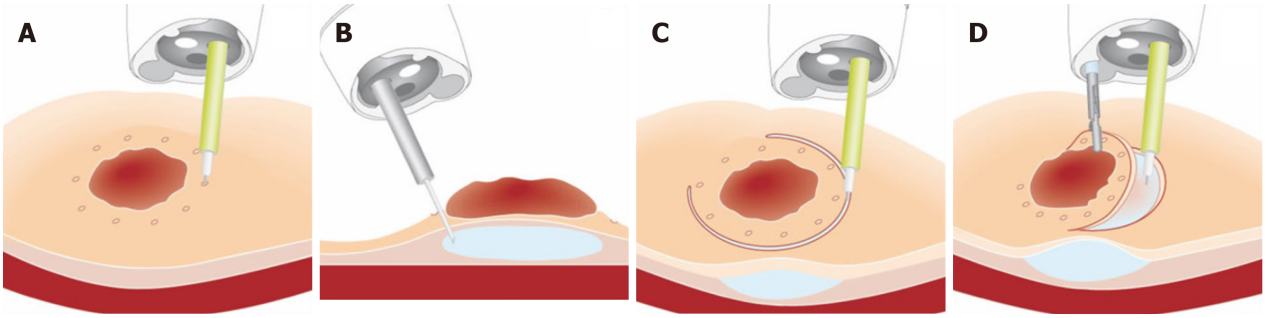


Figure 5 Endoscopic submucosal dissection using an additional working channel[172]. A: Lesion marked *via* usual working channel; B: Submucosal injection; C: Near-circumferential incision made; D: Lesion grasped for countertraction *via* additional working channel while dissection underway. Citation: Knoop RF, Wedi E, Petzold G, Bremer SCB, Amanzada A, Ellenrieder V, Neesse A, Kunsch S. Endoscopic submucosal dissection with an additional working channel (ESD+): a novel technique to improve procedure time and safety of ESD. *Surg Endosc* 2021; 35: 3506-3512. ©The Author(s) 2021. Published by Springer Open Access Article.

difficulty rating from endoscopists[179].

Spring and loop clip traction (using an S-O clip): This method uses the S-O clip (Figure 7) which is attached to a spring and then a loop of nylon[181,182]. One clip is attached to the edge of the lesion, while the other clip is attached to an area of opposing gastric wall to provide traction. Nagata[181] reported data from 140 patients of which 51 had spring and loop clip traction, with shorter procedure times and no difference in complication rates compared to conventional ESD[181]. The main limitations of this technique are the requirement for specific equipment (*i.e.*, the S-O clip) as well as only allowing one direction of traction once the clips are deployed.

Other clip methods: Various methods have been described employing readily available endoscopic equipment with clips to minimise cost. Yoshida *et al*[182] reported the efficacy of the 'dental floss clip' (DFC) traction device using dental floss tied to the proximal end of a clip before attaching the clip to the edge of the lesion, with the dental floss running externally to the endoscope[182]. There was no difference in procedure time overall, however in upper and middle greater curvature lesions DFC traction reduced procedure time by approximately 50%. In addition, the



Figure 6 'Endo-lifter' (Olympus-Tokyo, Japan)[192]. Citation: Harlow C, Sivananthan A, Ayaru L, Patel K, Darzi A, Patel N. Endoscopic submucosal dissection: an update on tools and accessories. *Ther Adv Gastrointest Endosc* 2020; 13: 2631774520957220. ©The Author(s) 2020. Published by Open Access Article.

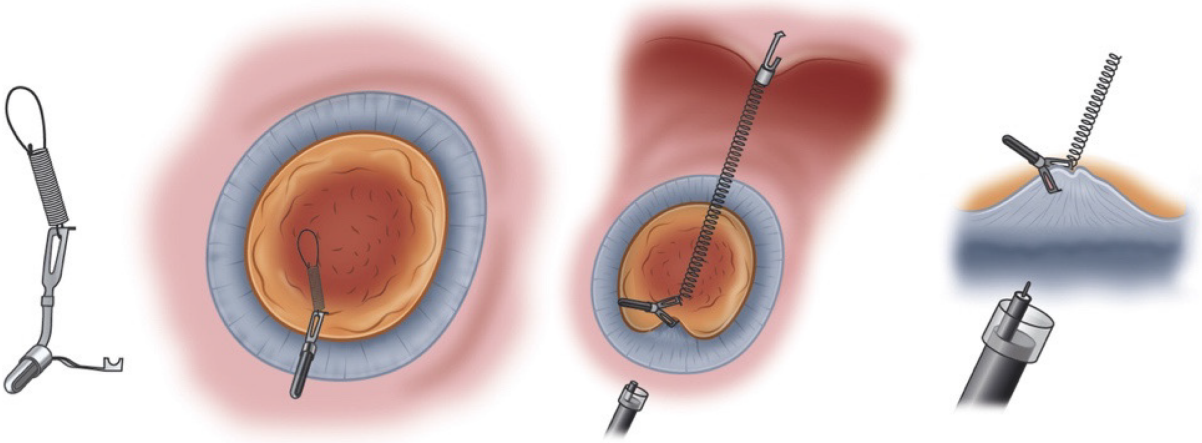


Figure 7 Spring and loop clip traction[193]. Citation: Nagata M, Fujikawa T, Munakata H. Comparing a conventional and a spring-and-loop with clip traction method of endoscopic submucosal dissection for superficial gastric neoplasms: a randomized controlled trial (with videos). *Gastrointest Endosc* 2021; 93: 1097-1109. ©The Author(s) 2021. Published by Open Access Article.

use of DFC traction reduced perforation rates compared to conventional ESD (0.3% *vs* 2.2%)[183]. Noda *et al*[183] added a polypectomy snare sheath external to the endoscope through which the thread (dental floss) was passed, which was then inserted along with the endoscope. The use of the sheath allows the thread to be moved without interference from the endoscope, as well as allowing both 'pull' and 'push' motions by advancing the entire sheath. In an 88 patient study comparing polypectomy snare sheath ESD with conventional ESD, this technique resulted in a reduction in procedure time and lower rates of significant bleeding[183]. Yoshida *et al*[184] also reported a reduction in procedure time by simplifying this technique to their 'clip and snare' technique: A snare with its sheath external to the endoscope is attached to the proximal end of a clip, then inserted and attached to the lesion[184]. Zhang *et al*[185] then modified the clip and snare technique (Figure 8), by using additional clips to attach the snare to the circumference of the lesion, allowing multi-focal lifting of the lesion, as well as placing clips attached to the snare on opposing gastric wall mucosa to alter the direction of traction[185].

Endoscopic full-thickness resection

FTR can either be performed free-hand, attempting to maintain the serosa although often resulting in macroscopic perforation, or device assisted (Figure 9) using an 'over-the-scope' clip[186,187]. While some data exist regarding the use of FTR for gastric GIST where successful resection rates are as high as 96.8%, there is no evidence

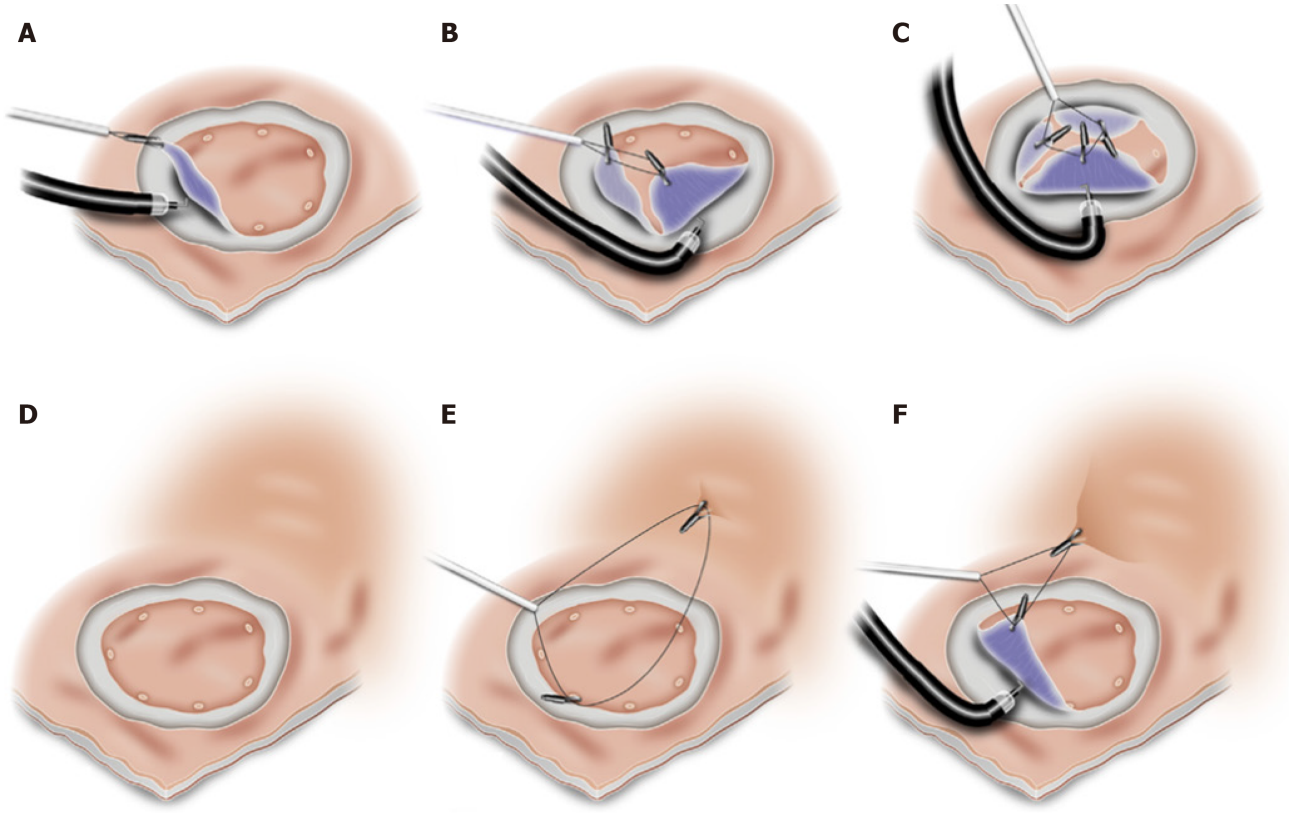


Figure 8 Modified endo-clip and snare[185]. A-C: Multiple clips used to provide multifocal traction; D-F: Clip applied to opposing gastric wall for countertraction. Citation: Zhang Q, Yao X, Wang Z. A modified method of endoclip-and-snare to assist in endoscopic submucosal dissection with mucosal traction in the upper GI tract. *VideoGIE* 2018; 3: 137-141. ©The Author(s) 2018. Published by Open Access Article.

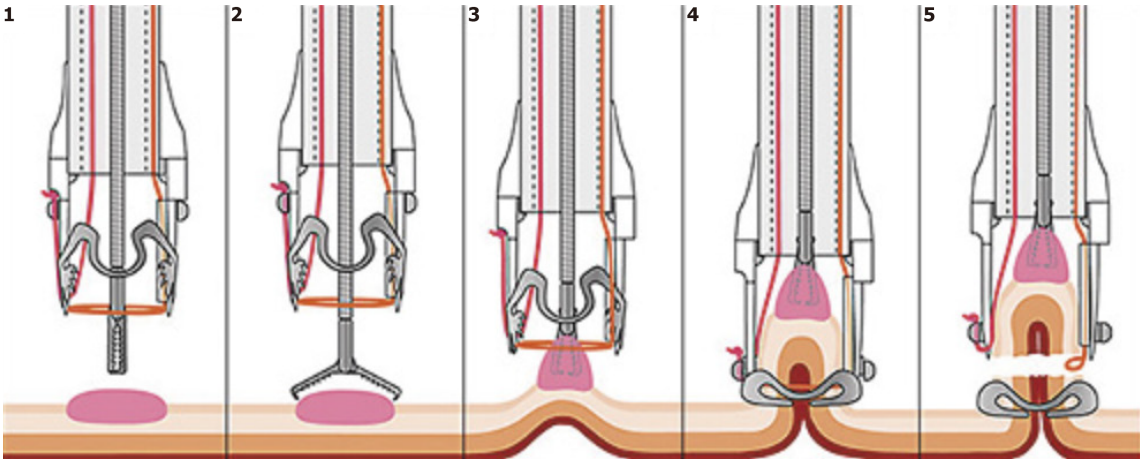


Figure 9 'Over-the-scope clip' (Ovesco, Germany) full-thickness resection[194]. Citation: Mão de-Ferro S, Castela J, Pereira D, Chaves P, Dias Pereira A. Endoscopic Full-Thickness Resection of Colorectal Lesions with the New FTRD System: Single-Center Experience. *GE Port J Gastroenterol* 2019; 26: 235-241. ©The Author(s) 2019. Published by Open Access Article.

regarding the use of FTR in EGCs[188]. Theoretical roles for gastric FTR could include treatment of recurrent lesions within EMR/ESD scar limiting repeat resection, or using FTR in combination with laparoscopic nodal resection in non-candidates for gastrectomy with lesions outside of expanded criteria for ESD. Chae *et al*[188] reported a successful case of FTR for an EGC with a positive horizontal margin after initial ESD which had resulted in severe fibrosis[188]. Cho *et al*[138] reported a series of 9 patients treated with FTR and laparoscopic lymph node resection who had lesions outside of expanded criteria, in whom lymph nodes were positive in only 1 case[138].

Robot-assisted ESD

The 'Master And Slave Transluminal Endoscopic Robot' (MASTER) was developed in an attempt to mitigate the technical difficulties commonly encountered in interventional endoscopy[189-191]. Initial feasibility studies were performed in 2010 in ex-vivo and in-vivo porcine stomachs, where 20 gastric lesions were successfully resected using MASTER assisted ESD with no difference in procedure time compared to conventional ESD[190]. Subsequently, a small case series was reported in 2012, using MASTER assisted ESD in 5 patients, demonstrating clear resection margins in all cases with no major complications[192]. More studies are required to further explore the role of robot-assisted ESD, however this technique will clearly be limited by the cost and availability of equipment, while proceduralists already successfully perform more conventional ESD techniques at a fraction of the cost.

CONCLUSION

Advances in endoscopic imaging and therapeutics have enhanced the detection, characterisation and treatment of precancerous gastric lesions and EGC, allowing for pre-emptive minimally invasive intervention at an early stage. Endoscopic treatment of precancerous lesions and EGCs not only reduces rates of advanced carcinoma, but also avoids the requirement for high-risk surgical interventions with associated short- and long-term complications. The significant disparity in gastric cancer outcomes between East Asian and Western regions reflects extensive endoscopist experience, exhaustive research and clear guidelines in East Asia. Accordingly, there is a requirement for high-quality collaborative data from Western populations to aid in the development of clear management algorithms. Globally there are a multitude of opportunities for further research in both endoscopic imaging and resection techniques to optimise lesion detection and outcomes of ER, as well as potentially establish new indications for ER into the future.

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REVIEW

Helicobacter pylori eradication: Exploring its impacts on the gastric mucosa

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Abstract

Helicobacter pylori (H. pylori) infects approximately 50% of all humans globally. Persistent *H. pylori* infection causes multiple gastric and extragastric diseases, indicating the importance of early diagnosis and timely treatment. *H. pylori* eradication produces dramatic changes in the gastric mucosa, resulting in restored function. Consequently, to better understand the importance of *H. pylori* eradication and clarify the subsequent recovery of gastric mucosal functions after eradication, we summarize histological, endoscopic, and gastric microbiota changes to assess the therapeutic effects on the gastric mucosa.

Key Words: *Helicobacter pylori*; Gastric mucosa; Histology; Endoscopic findings; Gastrointestinal microbiota; Eradication therapy

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Core Tip: Eradication of *Helicobacter pylori* (*H. pylori*) is important. Multiple gastrointestinal diseases and extragastric diseases would emerge if *H. pylori* infection persists, whereas they would improve after *H. pylori* eradication. Thus, *H. pylori* eradication produces dramatic changes in the gastric mucosa. This review highlights the most recent literature and presents a comprehensive evaluation about the impact of *H. pylori* eradication on the gastric mucosa.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) represents a type of Gram-negative microaerophilic bacterium with a helical shape, generally infecting humans in early childhood[1,2]. O'Connor *et al*[3] have generated a table with some of the latest epidemiological findings about *H. pylori* infection, whose rate remains high, especially in certain parts of China as well as some Eastern European and South American countries. *H. pylori* infects ~50% of the global population[4]. Some researchers have reported that *H. pylori* infection rate is associated with socioeconomic status, including educational resources and living conditions, indicating that elevated *H. pylori* prevalence is more likely to happen in underdeveloped countries[5,6]. *H. pylori* is transmitted *via* iatrogenic, fecal–oral, and oral–oral routes[7].

Gastrointestinal diseases develop if *H. pylori* infection persists, including acute and chronic gastritis, gastric and duodenal ulcers[8], gastric mucosa-associated lymphoid tissue lymphoma (MALToma)[9], and autoimmune gastritis (AIG)[10]. Several studies have reported that *H. pylori* infection plays a role in extragastric diseases, including immune thrombocytopenia, unexplained iron-deficiency anemia, and Alzheimer's disease[11-15]. Moreover, the World Health Organization has included *H. pylori* among group 1 carcinogens for its critical role in gastric cancer (GC) etiology[16,17].

Besides curing gastritis, complete eradication of *H. pylori* can permanently cure peptic ulcers[18] and induce MALToma regression[19]. Additional evidence also suggests that *H. pylori* eradication treatment decreases precancerous lesions[20] and successfully prevents GC development[21,22], even after resection of early GC[23]. There is an urgent need to clearly assess the importance and necessity of *H. pylori* eradication. Therefore, the purpose of this review is to examine the impact on the gastric mucosa of *H. pylori* eradication to better understand the importance of *H. pylori* eradication.

GASTRIC MUCOSAL CHANGES AFTER *H. PYLORI* ERADICATION

The gastric mucosa, the innermost layer of the stomach, consists of the epithelium, lamina propria, and muscularis mucosae, constituting three protective mucosal barriers. The most important barrier is called epithelial–bicarbonate barrier, the first line of defense of the gastric mucosa[24]. On the one hand, long-term *H. pylori* infection induces a sequence of histopathological changes, from gastritis (acute, chronic, and atrophic), intestinal metaplasia (IM), dysplasia, and ultimately to neoplasia according to the classical Correa sequence[25,26]. On the other hand, after anti-*H. pylori* therapy using antibiotics and proton pump inhibitors (PPIs)[27], the gastric mucosa undergoes various changes.

HISTOLOGICAL CHANGES UPON *H. PYLORI* ERADICATION

With *H. pylori* infection, the histological changes in the gastric mucosa, such as gastritis, are among the important and obvious manifestations. Evaluation of the extent of gastritis was proposed and revised based on the Sydney System[28] and/or the Updated Sydney System[29], comprising endoscopic and pathological findings. However, the *H. pylori* eradication efficiency can be also evaluated by histological indicators of activity (neutrophil polymorph density), inflammation (lymphocyte and plasma cell elevations), atrophy, and IM.

Changes of inflammation and activity

Regarding changes in histological indicators of gastric mucosal activity and inflammation, comparable trends of improvement have been reported[30-35]. Activity was improved in all studies. In addition, several studies have reported neutrophil

disappearance early after *H. pylori* eradication; consequently, activity score is considered a highly sensitive index for assessing *H. pylori* presence. Meanwhile, the inflammatory index PGII declines rapidly within 1–2 mo after successful *H. pylori* eradication[36,37]. Furthermore, inflammation is cleared at a significantly reduced rate, but with overt improvement[38].

Changes in atrophy and IM

Atrophic gastritis (AG) and IM are premalignant conditions for GC. It remains controversial whether *H. pylori* eradication reverses AG and IM.

Various parts of the stomach exhibit different histological recoverability. With a 1-year follow-up, Sung *et al*[34] carried out a study in 2000, screening 587 *H. pylori*-positive subjects, randomizing them to the omeprazole, amoxicillin and clarithromycin (n = 295) or placebo (n = 292), and indicating that GA and IM in the antrum and corpus could be alleviated by *H. pylori* eradication, as did other studies by Annibale *et al*[39] and Ohkusa *et al*[40]. However, a recent study performed by Sung *et al*[35] in 2020 corrected the above results, demonstrating that GA is improved significantly with radical treatment of *H. pylori* in the antrum and corpus, while IM did not follow the same trend. With 3 years of follow-up, our team previously assessed 197 *H. pylori*-infected patients, including 92 receiving *H. pylori* eradication therapy and 87 control patients, and found markedly decreased atrophy in individuals with successful *H. pylori* eradication[33]. However, Kang *et al*[41] found that AG was improved in the corpus but not in the antrum. Furthermore, Kodama *et al*[42] showed that atrophy was markedly reduced after *H. pylori* eradication, both in the antrum and corpus after 5–13 years of follow-up. At the same time, IM was significantly decreased in the corpus but not in the antrum, with no differences observed in the untreated group. With 10 years of follow-up, Kodama *et al*[43] evaluated the gastric mucosa at five points based on the Updated Sydney System, revealing that atrophy at every site in the stomach and IM in the lesser curvature of the corpus showed continuous and significant decreases. In addition, Hwang *et al*[44] prospectively assessed patients with a 10-year follow-up, demonstrating that AG and IM in the antrum and corpus were gradually alleviated and reached a point at which they were comparable to those of *H. pylori*-negative individuals. There are three meta-analyses[45-47] concerning improvements of AG and IM. The first[45] assessed the long-term impact of *H. pylori* eradication on histological features in the stomach, and demonstrated that eradicating *H. pylori* improved atrophy but not IM, a finding similar to that of another meta-analysis[46]. However, Kong *et al*[47] reported that IM improvement only occurred in the gastric antrum and not in the corpus.

The discrepant responses of AG and IM to *H. pylori* eradication may have several reasons. On the one hand, the methods of histological assessment of biopsy specimens, sample sizes, and amounts of biopsy specimens are different across studies. On the other hand, progression from AG to serious AG, IM, and GC takes decades, indicating that a longer follow-up period could better mimic the actual situation[48,49]. Moreover, different risk factors for AG and IM, such as bile reflux, other bacterial infections, age, and dietary structure, could also influence the final results[33,50].

Different follow-up times result in different recoverability degrees of AG and IM. Since follow-up is tightly associated with improvement data in the majority of studies, follow-up times were divided into three groups for further assessment of AG and IM, including short (< 3 years), medium (3–10 years), and long (≥ 10 years) terms (Table 1). Activity and inflammation improvements following *H. pylori* eradication were consistent. However, whether AG and IM can be completely cured upon *H. pylori* eradication remains debatable. It is worth noting that a research team in Colombia conducted a large trial with long-term follow-up in the 1990s. After 6 years[51], 12 years[32], 16 years[52], and 20 years[53], the results indicated that *H. pylori* infection increased histological progression, and anti-*H. pylori* treatment significantly induced histological improvement and disease regression, and reduced progression of precancerous lesions of GC. Therefore, AG could be reversed, and even IM, with prolonged follow-up.

The above findings suggest that *H. pylori* eradication improves AG and IM, and anti-*H. pylori* treatment confers long-term benefits in decreasing the progression of precancerous lesions. The earlier the *H. pylori* eradication, the greater the benefits.

CHANGES IN ENDOSCOPIC FINDINGS AFTER *H. PYLORI* ERADICATION

Endoscopy is an important gastrointestinal examination method. The Kyoto Classi-

Table 1 Major features of the eight trials examining for histological parameters

Ref.	Study arm, *n*		Follow-up, yr	Medication	Methods	Histologic parameter											
	Eradicated	Not eradicated			1 = OS	AG						IM					
					2 = RCT	Antrum			Corpus			Antrum			Corpus		
						Before	After	*P* value	Before	After	*P* value	Before	After	*P* value	Before	After	*P* value
Sung *et al* [34]	226	245	1	OAC	2	0.64 ± 0.78	0.70 ± 0.82	*P* = 0.627	0.06 ± 0.31	0.02 ± 0.18	*P* = 0.682	0.78 ± 0.98	0.61 ± 0.94	*P* = 0.014	0.04 ± 0.32	0.06 ± 0.30	*P* = 0.391
Annibale *et al*[39]	25	7	0.5	BAM	1	0.56 ± 0.24	0.5 ± 0.2	NS	1.64 ± 0.11	1.36 ± 0.18	NS	0.58 ± 0.25	0.53 ± 0.23	NS	0.52 ± 0.13	0.76 ± 0.16	NS
Ohkusa *et al* [40]	115	48	1-1.25	PPI/A/C	1	0.8 ± 1	At 1–3 mo: 0.8 ± 1	*P* > 0.2	0.5 ± 0	At 1–3 mo: 0.3 ± 0	*P* = 0.020	0.7 ± 0	At 1–3 mo: 0.6 ± 0	*P* = 0.14	0.0 ± 0.0	At 1–3 mo: 0.2 ± 0	*P* = 0.022
							At 12–15 mo: 0.9 ± 1	*P* = 0.15		At 12–15 mo: 0.2 ± 0	*P* = 0.001		At 12–15 mo: 0.4 ± 0	*P* < 0.001		At 12–15 mo: 0.1 ± 0	*P* > 0.2
Lu *et al*[33]	92	62	3	O/LAC	1	1.25 ± 0.44	0.97 ± 0.83	*P* < 0.01	NA	NA	NA	0.64 ± 0.76	0.73 ± 0.77	NS	NA	NA	NA
Kang *et al* [41]	210	16	3	PPI/A/C	1	0.85 ± 0.06	1 yr: 0.83 ± 0.06	NS	0.70 ± 0.07	1 yr: 0.42 ± 0.06	*P* < 0.001	0.91 ± 0.07	1 yr: 0.83 ± 0.06	NS	0.60 ± 0.07	1 yr: 0.54 ± 0.06	NS
	54	16	3	PPI/A/C	1	0.96 ± 0.14	3 yr: 1.32 ± 0.20	NS	0.91 ± 0.20	3 yr: 0.45 ± 0.15	*P* = 0.033	1.02 ± 0.14	3 yr: 1.29 ± 0.14	NS	0.68 ± 0.15	3 yr: 0.83 ± 0.14	NS
Kodama *et al* [42]	118	21	8.6	PPI/A/C	1	1.60 ± 0.09	1.02 ± 0.08	*P* < 0.001	0.71 ± 0.10	0.02 ± 0.02	*P* < 0.001	0.60 ± 0.11	0.43 ± 0.09	NS	0.17 ± 0.12	0.00 ± 0.00	*P* < 0.05
Kodama *et al* [43]	176	21	10	PPI/A/C	1	AI: 1.39 ± 0.07	6 yr: 0.90 ± 0.09	*P* < 0.05	B1: 1.08 ± 0.08	1 yr: 0.78 ± 0.11	*P* < 0.05	A1: 1.14 ± 0.10	NA	NS	B1: 0.97 ± 0.09	6y: 0.42 ± 0.17	*P* < 0.05
						A2: 1.39 ± 0.06	1 yr: 1.06 ± 0.08	*P* < 0.01	B2: 0.52 ± 0.06	0.6 mo: 0.29 ± 0.07	*P* < 0.05	A2: 0.50 ± 0.07	NA	NS	B2: 0.13 ± 0.04	NA	NS
						IA: 1.51 ± 0.08	1 yr: 1.24 ± 0.09	*P* < 0.05				A1: 1.04 ± 0.09	NA	NS			
Hwang *et al* [44]	442	91	10	PPI/A/C E/A/C/M	1	*n* = 178	*n* = 89 (50.0)	*P* = 0.002	*n* = 105	*n* = 72 (68.8)	*P* = 0.01	*n* = 221	*n* = 45 (20.4)	*P* = 0.002	*n* = 142	*n* = 31 (21.8)	*P* = 0.01

A: Amoxicillin; A1: Lesser curvature of the antrum; A2: Greater curvature of the antrum; B1: Lesser curvature of the corpus; B2: Greater curvature of the corpus; B: Bismuth subcitrate; C: Clarithromycin; E: Esomeprazole; GA: Gastric atrophy; IA: Lesser curvature of the angulus; IM: Intestinal metaplasia; L: lansoprazole; M: Metronidazole; NA: Not applied; NS: Not significant; O: Omeprazole; OS: Observational study; PPI: Proton pump inhibitor; RCT: Randomized controlled trial; data are presented as *n* (%) or the median ± standard deviation or mean ± SE/median.

fication of Gastritis, categorizing *H. pylori* infection into three phases (non-gastritis, active gastritis, and inactive gastritis[54]), was proposed to better assess the status of *H. pylori* infection and GC risk by endoscopy[55] (Figure 1). In a healthy stomach, an easily detectable feature, non-gastritis, was the regular arrangement of collecting venules (RAC), featured as small red spots on the mucosal surface[56,57]. However, after being infected with *H. pylori*, the stomach was characterized as irregular arrangement or absence of the so-called collecting venules[58]. AG after infection by *H. pylori* presents with diffuse redness, spotty redness, mucosal edema, and enlarged folds. This phenomenon can decrease and disappear after *H. pylori* eradication[59-61]. In addition, with *H. pylori* eradication, nodular gastritis (NG), whose endoscopic character is "goose flesh" in the antrum, can also disappear with the passage of time [62,63].

After a period of *H. pylori* infection, AG turns into inactive gastritis upon eradication therapy or spontaneously disappears because of advanced atrophy, featuring map-like redness, and flat or depressed erythematous tumors, which is the characteristic change of AG after *H. pylori* eradication, *i.e.*, nonatrophied areas dissipate the inflammation, and the atrophied areas are relatively red compared to the nonatrophied areas. Using white-light imaging (WLI) and linked color imaging (LCI), Majima *et al*[64] found that map-like redness is closely associated with GC occurrence upon effective *H. pylori* eradication. Another study also revealed map-like redness upon *H. pylori* eradication as the sole predictive factor for metachronous cancer[65]. With *H. pylori* infection, atrophic change expands from the antrum to the fundus, and is improved after eradication[66,67]. Another characteristic was described as mottled patchy erythema (MPE) after *H. pylori* infection, showing many flat/slightly depressed erythematous lesions detected by white light endoscopy, and highly predicting the impact of *H. pylori* eradication.

The typical endoscopic finding of IM is mixed patchy pink and pale mucosal areas surrounding grayish slightly elevated plaques generating an irregular, uneven surface. Moreover, villus-like structures, whitish mucosa, and rough mucosal surface can help diagnose IM by endoscopy[68,69]. In addition, endoscopic IM contributes to recognition of current and past *H. pylori* infections, similar to endoscopic atrophy[70]. *H. pylori* eradication reduces the development of hyperplastic polyps (HPPs); either sessile or pedunculated polyps result from *H. pylori* infection[71]. Gastric xanthoma (GX) is a typical endoscopic manifestation of *H. pylori* infection that persists upon *H. pylori* eradication, showing one or more yellowish well-delineated nodules or plaques of 1–10 mm in diameter[72]. However, GX may be a precancerous lesion of GC[72,73]. After treatment with PPI, the endoscopic phenomena of multiple white elevated lesions and cobblestone-like mucosa became more evident in comparison with PPI nonusers[74].

Overall, endoscopic features represent additional indexes for evaluating *H. pylori* therapy for efficacy. Atrophy, IM, HPPs, and fundic gland polyps are detected in active and inactive gastritis. In addition, atrophy boundaries are unclear with map-like redness observed upon *H. pylori* eradication[75]. However, endoscopic atrophy and IM may show no rapid improvement[76,77], and prolonged follow-up is required for detecting gastric mucosal changes endoscopically following *H. pylori* eradication[78].

EFFECT OF *H. PYLORI* ERADICATION THERAPY ON GASTRIC MICROBIOTA

There are many microorganisms in the human stomach, constituting alongside *H. pylori* the so-called gastric microbiota[79], whose balance and stability are indispensable for normal gastric mucosal digestion and metabolism. With more advanced techniques, such as culture-free molecular methods (*e.g.*, 16S rDNA sequencing), the human stomach is currently known to host multiple resident microbes. Based on such techniques, many reports have shown that *H. pylori*-negative individuals have a greatly diverse gastric microbiome with four dominating phyla, including Proteobacteria (including *H. pylori*), Firmicutes, Bacteroidetes, and Actinobacteria; the commonest genera are *Streptococcus*, *Lactobacillus*, and *Propionibacterium*[80-85].

Upon *H. pylori* infection, changes in gastric microorganisms arise, including gastric microbial diversity, composition, and predictive pathways[86], leading to various diseases[87-89]. Generally, colonization by *H. pylori* is associated with significantly reduced alpha and beta diversities (representing inter-sample and in-sample diversities, respectively)[90-92]. Additionally, several studies have revealed that *H.*

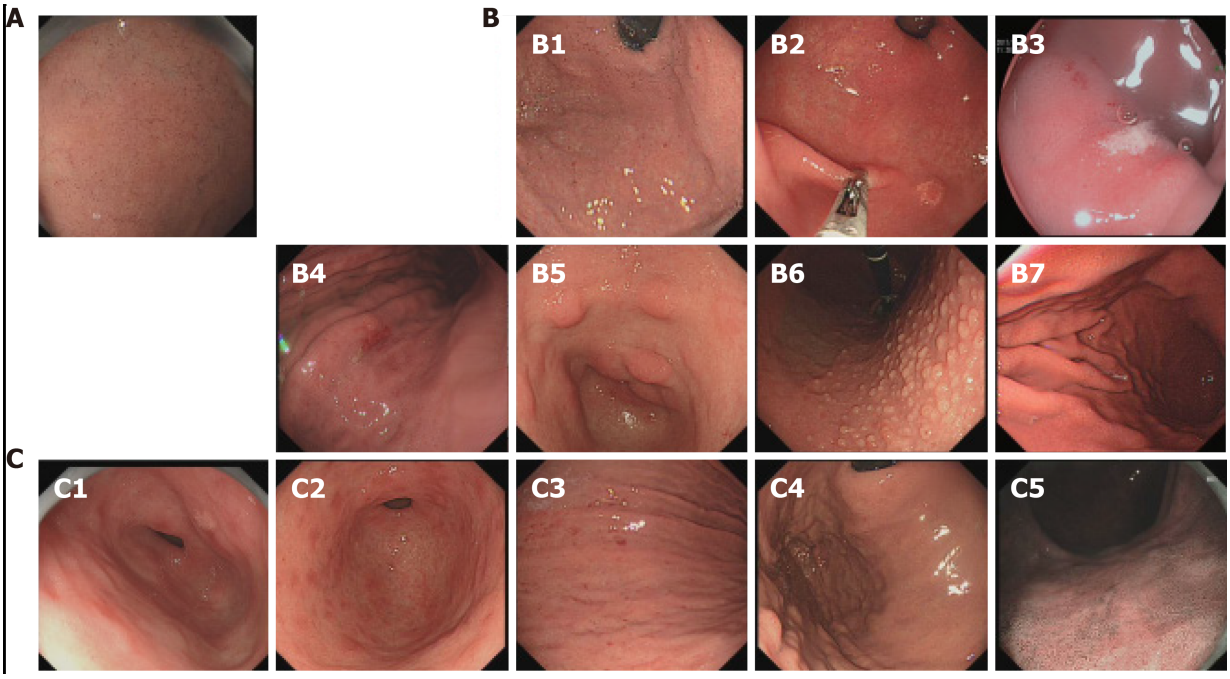


Figure 1 Endoscopic features for *Helicobacter pylori* infection. A: Normal gastric mucosa. Regular arrangement of collecting venules is seen; B: Infected gastric mucosa. B1: Spotty redness; B2: Gastric xanthoma; B3: Erosion; B4: Multiple redness and erosion; B5: Hyperplastic polyp; B6: Nodular gastritis; B7: Intestinal metaplasia; C: Gastric mucosa after eradication. C1: Patchy redness; C2: Map-like redness; C3: Redness; C4: Atrophy; C5: Intestinal metaplasia.

pylori-infected individuals have different community structures in comparison with their *H. pylori*-negative counterparts[93-96]. Compositionally, Proteobacteria often dominate the gastric mucosa upon *H. pylori* infection, becoming the single most abundant bacteria and almost reaching 90% abundance at the phylum level, while other phyla (Actinobacteria, Bacteroidetes, Firmicutes, and Fusobacteria) show reduced numbers[82,90,92-94,96,97].

After anti-*H pylori* treatment, the gastric microbiome undergoes major reshaping (Table 2). Mounting evidence indicates that gastric microbial diversity markedly increases upon effective *H. pylori* eradication but does not improve if treatment fails [35,82,86,93,98,99]. Recovery may take some time as microbial diversity increases gradually from week 0 to weeks 6 and 26[86]. Additionally, alpha diversity can regain the level of uninfected individuals following effective eradication[98]. Although the community structure can also be partly restored upon *H. pylori* eradication, whether in post-eradication groups it can be restored to that of healthy control groups appeared to be age related. Specifically, several studies indicated that the adult specimens from 6 mo after successful treatment still showed altered community structure *vs* the negative control group[98], while others recruiting children reported the close community structures between the eradication and *H. pylori*-negative groups at 4 wk post-therapy [99] and the restored gastric microbiota composition in individuals administered with anti-*H. pylori* therapy at 2 mo post-treatment[82]. We believe that in adult patients, further research is needed to see whether the recovery in microbial composition can be observed over a longer observation period.

Compositionally, the relative abundance of *H. pylori* starkly decreases post-treatment, although it remains the dominant bacterium[86,93]. Meanwhile, Actinobacteria, Firmicutes, Bacteroidetes, and Fusobacteria are significantly enriched after successful eradication[82,90,93,98]. At the genus level, the probiotics *Lactobacillus* and *Bifidobacterium* are markedly increased post-therapy[86]. Functional analysis was performed in multiple studies[82,86,98]. The activities of disease-associated categories in *H. pylori* infection (lipopolysaccharide biosynthesis, bacterial motility proteins, *etc*.) were more pronounced[82,98]. In addition, the metabolic pathways (protein digestion and absorption, gastric acid secretion, and carbohydrate digestion and absorption) in the presence of *H. pylori* were downregulated[100]. After eradication therapy, these functions might be partly restored[86].

H. pylori infection is associated with reduced bacterial diversity and causes a shift in bacterial structure. Clearance of *H. pylori* significantly increases bacterial diversity. The relative abundance of *Helicobacter* decreases after therapy, while other phyla are increased, partly restoring bacterial structure and improving microbiota functions,

Table 2 Major features of five meta-analyses

Ref.	Year	Total No. of study	Eradication group			Control group			OR/RR	95%CI
			Total No.	Total events	Incidence rate	Total No.	Total events	Incidence rate		
Sugimoto *et al*[118]	2020	4RCTs	2731	73	2.7%	2733	49	1.80%	0.67	0.47–0.96
Sugano *et al*[119]	2019	32	16301	316	1.90%	14805	535	3.60%	0.46	0.39-0.55
Doorakkers *et al* [120]	2016	8 Cohort	12899	119	0.90%	18654	208	1.10%	0.46	0.32-0.66
Chen *et al*[121]	2016	8RCTs	3992	74	1.90%	3962	116	2.90%	0.64	0.48-0.85
Ford *et al*[104]	2014	6RCTs	3294	51	1.60%	3203	76	2.40%	0.66	0.46-0.95

RR: Risk ratio; OR: Odds ratio; 95% C: 95% confidence interval.

such as metabolism.

CHANGES IN GC AFTER *H. PYLORI* ERADICATION

Many studies have confirmed that *H. pylori* infection is the main etiological agent of GC[101,102], whose risk can be reduced by *H. pylori* eradication[103-108].

To explore this, Wong *et al*[109] performed a study demonstrating that GC incidence rates were comparable in the treatment and placebo groups (7 cases in either group), which may have been due to a underpowered design despite the 7.5-year follow-up of 1630 participants. However, with the follow-up time gradually extended, the incidence rates of GC in both groups gradually showed differences. Another study demonstrated significantly decreased GC incidence after 6 years of follow-up after *H. pylori* eradication, and the standardized incidence ratio (SIR) was 1.62 in the initial 5 years but was reduced thereafter to reach 0.14[21]. A Swedish cohort study found significantly decreased risks of gastric adenocarcinoma and non-cardia gastric adenocarcinoma upon cure of *H. pylori* infection (SIRs were 8.65 in 1–3 years, 2.02 in 3–5 years, and 0.31 in 5–7.5 years)[22,109]. After *H. pylori* treatment, the risk was 39% lower over an extended follow-up of 15 years and 52% over an extended follow-up of 22 years among individuals with *H. pylori* eradication compared with those showing persistent infection, whereas there was no difference during the initial 7.3-year follow-up[20,110,111]. Having a first-degree relative with diagnosed GC doubles or triples GC risk[112]. In *H. pylori*-infected individuals with a first-degree relative diagnosed with GC, eradication of *H. pylori* also reduces GC risk[106,113]. A South Korean study utilized a prospective randomized design (832 and 844 in the cure and placebo groups, respectively, of first-degree relatives of GC cases). GC risk was reduced by 55% after *H. pylori* eradication *vs* the placebo group, with an average follow-up of 9.2 years. Of note, GC risk was 73% lower upon *H. pylori* eradication compared with the placebo group.

GC, as the end point of gastric disease, is also inextricably linked to *H. pylori*. Choi and collaborators[114] found that *H. pylori* eradication had no significant relationship with metachronous GC (MGC) incidence within an average follow-up of 3 years, whereas *H. pylori* eradication markedly reduced MGC incidence with a median follow-up duration of 71.6 mo[115]. A recent randomized trial involving early GC cases (a population that usually has severe atrophic alterations in the gastric mucosa) demonstrated that treating *H. pylori* infection reduced MGC risk by half[106]. A similar effect was also reported in another Chinese trial[116]. Successful eradication therapy cannot completely eliminate the development of GC. Take *et al*[117] performed a retrospective cohort trial in Japan, including 2737 patients treated for *H. pylori* infection with yearly endoscopic follow-up for 21.4 years. The degree of atrophy was related to a high yearly risk of GC. They also found an elevated risk of diffuse-type GC in individuals with mild to moderate gastric atrophy at baseline. The above findings suggest that endoscopic monitoring for GC should continue beyond 10 years post-*H. pylori* eradication regardless of the degree of gastric mucosal atrophy at the time of eradication treatment[117].

Several meta-analyses have demonstrated that the risk of GC is correlated with *H. pylori* eradication (Table 3)[104,118-121]. One meta-analysis including six randomized studies involving healthy, asymptomatic participants with *H. pylori* infection showed that GC risk was about 34% less after treatment compared with the control group [104]. Another meta-analysis also showed a reduced incidence of GC upon eradication therapy compared with control patients (pooled incidence rate ratio = 0.54)[122]. Sugano *et al*[119] and Doorakkers *et al*[120] reported that the lower odds ratio/relative risk was 0.46. A further meta-analysis demonstrated that no matter how varied the countries, conditions at baseline, and follow-up periods among studies, *H. pylori* eradication effectively reduces GC incidence. Consistent with the prediction, long-term (≥ 5 years) follow-up showed greater effects in reducing GC upon *H. pylori* eradication compared with shorter follow-up periods (< 5 years)[119]. This was consistent with other meta-analyses[104,118,120-122]. Thus, the above meta-analyses provided further robust evidence of the effect of eradication treatment.

Precancerous lesions are closely associated with GC. Consequently, whether and when *H. pylori* eradication reverses precancerous tumors has attracted increasing attention. Kiriyama *et al*[123] and Wong *et al*[109] have reported that eradicating *H. pylori* did not reverse mucosal injury in IM to yield a normal gastric mucosa or prevent GC development, indicating a histological point of no return. In agreement, others have indicated that GC progression continues following *H. pylori* eradication[109,124]. However, the Taipei global consensus and Matsu Islands consensus proposed that eradicating *H. pylori* reduces GC risk[107,108], which may be due to treatment effects before a certain point for preventing GC.

In general, GC risk in *H. pylori*-infected patients is increasing. A large number of studies have shown that *H. pylori* eradication can reduce the incidence of not only GC, but also MGC. In both small and large studies (community, region, or country) examining young and old individuals, and even first-degree relatives of patients, eradication of *H. pylori* results in long-term benefits.

DISCUSSION

H. pylori infection induces a sequence of histological changes, especially AG and IM. A histological classification system (Figure 2A) was proposed by an international group of gastroenterologists and pathologists, to grade gastritis into stages with corresponding cancer risk in individual patients, termed the Operative Link on Gastritis Assessment (OLGA) scale[125]. However, disease severity and extent in OLGA are primary parameters, which leads to low interobserver agreement. Therefore, a staging system based on IM (Operative Link on Gastric Intestinal Metaplasia Assessment, OLGIM; Figure 2B) was proposed to assess the degree of IM and GC risk in 2010[126]. However, some individuals potentially at high risk of GC may be overlooked[127]. Therefore, the combination of OLGA and OLGIM more accurately predicts GC risk. Meanwhile, the AI system using deep learning (especially convolutional neural networks; CNNs) has been applied in gastroenterology[128-131]. For example, studies have reported the usefulness of CNN-based AI systems for diagnosing *H. pylori* infection and timely detecting gastric neoplasms[129,131,132].

Previous studies have found that only a small number of patients with *H. pylori* infection develop GC eventually, but *H. pylori* is one of the main causes of GC. The high risk of GC emphasizes the need for early detection and proper treatment of *H. pylori* infection. Along with standard endoscopy, new endoscopic techniques, such as magnifying endoscopy[133], endocytoscopy[134,135], magnifying narrow-band imaging (M-NBI)[136], I-Scan[137], endomicroscopy[138], and LCI[139-141], can be used to detect *H. pylori* infection. Magnifying endoscopy allows the structure of the mucosa and the subepithelial capillary network around the gastric fovea to be observed in detail. As a novel ultra-high magnification technology, endocytoscopy can recognize gastric mucosal minimal changes[134,135]. Moreover, the NBI system and I-Scan are also the recent developments in computed virtual chromoendoscopy imaging [137]. The diagnostic accuracy of M-NBI endoscopy for gastritis and magnifying I-Scan for *H. pylori* infection was 96.1% and 94.0%, respectively[136,137]. Currently, a novel imaging mode under blue laser endoscopy, LCI, plays an important role in endoscopic diagnosis of active *H. pylori* infection or distal gastric disease, through its enhanced slight differences in mucosal color[139-141]. With the assistance of computer-aided diagnosis (CAD) systems, LCI-CAD can effectively assess the gastric mucosal status of uninfected, currently infected, and post-*H. pylori* eradication patients[142-144].

Table 3 Studies on gastric microbiota alteration after eradication

Ref.	Year	Total subjects	Follow-up time	Age	Regimen	Study group		Main outcomes
				1 = Adults 2 = Children	1 = TT for 7-14 d 2 = QT for 10-14 d	HEG	*H. pylori* (-)	
Li *et al*[93]	2017	33	Day 0 and week 9	1	1	17	16	Bacterial diversity increased and the relative abundance of *Helicobacter* decreased, while the relative abundance of other phyla increased
Serrano *et al*[99]	2019	16	Day 0 and month 2	2	1	11	5	Bacterial diversity increased and the structures of the uninfected group were restored
Guo *et al* [98]	2020	164	Day 0 and month 6	1	2	115	49	Bacterial diversity returned to the level of the control group. The structure of the bacteria was different after treatment compared to the control group. Microbiota functional capacities were changed
He *et al* [86]	2019	17	Weeks 0, 6, and 26	1	2	10	NA	Bacterial diversity increased and structure and microbiota functional capacities were changed
Miao *et al* [82]	2020	55	Day 0 and week 4	2	1, 2 and STP	11	8	Diversity was similar compared to the control group. The bacterial structure became close to controls
Sung *et al* [35]	2020	102	Day 0 and 1 year	1	1	102	NA	Bacterial diversity increased and structure was changed

HEG: *Helicobacter pylori* eradication group; *H. pylori*: *Helicobacter pylori*; NA: Not applicable; QT: Quadruple therapy; TT: Triple therapy; STP: Sequential therapy with proton pump inhibitor and amoxicillin.

In this review, we describe the changes in gastric histology, endoscopic appearances, gastric microbiota, and decreased risk of GC and MGC[108]. Dyspeptic symptoms, AIG, and recurrence of peptic ulcer disease significantly declined after eradication of *H. pylori*. The risk of synchronous GC after endoscopic resection of early GC was also reduced. Many extragastric disorders, such as iron deficiency anemia, MALToma, and idiopathic thrombocytopenic purpura, were also associated with the presence of *H. pylori* and they were improved after eradication of *H. pylori*[8,9,10,145,146]. Therefore, consensus reports recommend eradication of *H. pylori* in infected patients, decreasing the risk of these diseases[38,147].

However, there are some potential concerns for *H. pylori* therapy due to the significantly increased antibiotic (particularly metronidazole and clarithromycin) resistance rates for *H. pylori*[148,149], and development of novel and alternative antimicrobial agents specific for *H. pylori* is urgent. These approaches are broadly divided into two main categories: (1) Novel synthetic treatment, which includes new classes of antimicrobial peptides (AMPs) and small molecule inhibitors; and (2) natural treatment options, which include the use of probiotics and phytotherapy to treat *H. pylori* infection. First, AMPs play a pivotal role in the innate immune responses to *H. pylori* in humans. AMPs can be roughly divided into nine categories: Pexiganan, tilapia piscidins, epinecidin-1, cathelicidins, defensins, bicarinalin, odorranain-HP, PGLa-AM1, and bacteriocins[150]. Among them, cathelicidins and defensins, both secreted by epithelial cells of many tissues, exhibit the key therapeutic potential[151]. SQ109, a typical representative of small molecule inhibitors for treating *H. pylori* infection, displays robust thermal and pH stability, induces low/no spontaneous drug resistance, and shows anti-*H. pylori* superiority over metronidazole and amoxicillin [152]. Second, adjuvant probiotics and phytotherapy therapy are designed to increase the eradication rate of *H. pylori* and reduce the adverse effects of treatment[153,154]. Phytotherapy, including herbs and spices, cruciferous vegetables, Korean red ginseng and green tea, and extracts of oils, resveratrol, and beta-carotene, is another naturopathic therapy. Specifically, herbal-based therapies, one of the most popular forms of phytotherapy, can act as anti-inflammatory agents to treat *H. pylori* infection[155]. Nevertheless, the active component for the majority of agents and the molecular mechanism of inhibition against *H. pylori* remain unknown. After the eradication of *H. pylori*, the risk of gastroesophageal reflux disease is increased due to the restoration of gastric acid secretion[156,157]. Alterations in gut microbiota might decrease the secretion levels of insulin, and fasting glucose, total cholesterol, and triglyceride were reduced after *H. pylori* eradication[148,158]. However, the findings remain contro-

A

Atrophy score		Corpus			
		No atroph (score 0)	Mild atrophy (score 1)	Moderate atrophy (score 2)	Severe atrophy (score 3)
Antrum (Including incisura angularis)	No atroph (score 0)	Stage 0	Stage I	Stage II	Stage II
	Mild atrophy (score 1)	Stage I	Stage I	Stage II	Stage III
	Moderate atrophy (score 2)	Stage II	Stage II	Stage III	Stage IV
	Severe atrophy (score 3)	Stage III	Stage III	Stage IV	Stage IV

B

IM score		Corpus			
		No IM (score 0)	Mild IM (score 1)	Moderate IM (score 2)	Severe IM (score 3)
Antrum (Including incisura angularis)	No IM (score 0)	Stage 0	Stage I	Stage II	Stage II
	Mild IM (score 1)	Stage I	Stage I	Stage II	Stage III
	Moderate IM (score 2)	Stage II	Stage II	Stage III	Stage IV
	Severe IM (score 3)	Stage III	Stage III	Stage IV	Stage IV

Figure 2 Operative link on gastritis assessment staging system (A) and operative link on gastric intestinal metaplasia assessment (B) staging system. IM: Intestinal metaplasia; OLGA: Operative link on gastritis assessment staging system; OLGIM: Operative link on gastric intestinal metaplasia assessment.

versial and further well-designed randomized trials are warranted to clarify the impact of *H. pylori* eradication on metabolic parameters.

More significantly, the relative immutability of IM is of concern, as the condition carries a high GC risk not only in the presence of *H. pylori* infection, but also after *H. pylori* eradication. In other words, GC can still develop even after successful eradication in the presence of IM[159]. Previous studies have indicated that the detection of map-like erythema, a histological indicator of IM, is correlated with a high risk of GC development after *H. pylori* eradication[65]. Even worse, the eradication therapy can cause some characteristics, such as a gastritis-like appearance, resulting in a difficult diagnosis of GC[160-162]. This is why post-eradication status should be distinguished from *H. pylori* negativity.

CONCLUSION

Whether *H. pylori* eradication confers long-term benefits has been debated for a long time. Obviously, eradication of *H. pylori* is more important, because the disadvantages can be avoided based on clinical experience and continuous technological development. More importantly, *H. pylori* eradication offers lifelong benefits, and the earlier it is eradicated, the better. In addition, more sensitive and accurate tools can be developed to detect *H. pylori* infection in the early and post-eradication stages. This could be a promising area of research.

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MINIREVIEWS

Molecular advances in pancreatic cancer: A genomic, proteomic and metabolomic approach

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) represents a challenging pathology with very poor outcomes and is increasing in incidence within the general population. The majority of patients are diagnosed incidentally with insidious symptoms and hence present late in the disease process. This significantly affects patient outcomes: the only cure is surgical resection but only up to 20% of patients present with resectable disease at the time of clinical presentation. The use of "omic" technology is expanding rapidly in the field of personalised medicine - using genomic, proteomic and metabolomic approaches allows researchers and clinicians to delve deep into the core molecular processes of this difficult disease. This review gives an overview of the current findings in PDAC using these "omic" approaches and summarises useful markers in aiding clinicians treating PDAC. Future strategies incorporating these findings and potential application of these methods are presented in this review article.

Key Words: Pancreatic ductal adenocarcinoma, Pancreatic adenocarcinoma; Genomic; Proteomic; Metabolomic

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Core Tip: Treatment for pancreatic ductal adenocarcinoma is limited by the severity of the pathology, limited biomarkers and late presentation of patients. Utilising genomic, proteomic and metabolomic research into pancreatic ductal adenocarcinoma has provided insight into understanding the disease process as well as providing suitable markers of diagnosis and treatment to improve clinical outcomes.

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INTRODUCTION

Pancreatic adenocarcinoma or pancreatic ductal adenocarcinoma (PDAC) is an aggressive malignancy with a 1-year and 5-year survival rate of 24% and 9% respectively and make up the majority of pancreatic cancers (PC) (85%) with others arising from the endocrine tissue of the pancreas[1]. According to the GLOBOCAN statistics in 2020, PC were the 12th most commonly diagnosed cancer in the world with the incidence increasing with the Human Development Index; the highest cumulative risks being in North America and Europe[2]. Unfortunately, developments in treatment have not progressed as rapidly as other cancers and PC are projected to become the second leading cause of cancer related death in the United States by 2030[3]. Majority of PDAC cases are diagnosed at a late stage owing to the lack of clinical features early on in its course. Currently the only curative option is through surgical resection, but only roughly 20% of all cases are operable at the time of diagnosis, with limited chemotherapy and radiotherapy options at present[4]. However, with the emergence of "omics" research, new advances have been made in improving the treatment of PC.

GENOMICS, PROTEOMICS AND METABOLOMICS

The field of "omics" research refers to the use of high throughput technologies to globally analyse a biological system at the molecular level. This takes place on multiple "levels" depending on the nature of the molecules being studied i.e. genetic material, proteins and metabolites[5].

The first of these fields to emerge was genomics, with the goal of characterising the genome and the variations of its structure and expression leading to pathogenesis[5]. Cancer being essentially a disease of the genome, occurring through the accumulation of genetic mutations, the insights gleaned from such analysis is invaluable for cancer research[6].

With the advent of next-generation sequencing [whole-genome sequencing (WGS), whole-exome sequencing (WES) and RNA sequencing], this can now be done quicker and more accurately than traditional methods[7]. WES, which sequences all protein coding exons, is more abundantly utilised as it is more accurate and relatively less expensive compared to WGS, which also has the issue of complex data analysis and interpretation[6].

At the protein level, proteomic studies identify and quantify the proteome of a biological system, their interactions and post-translational modifications[5]. Proteomics technologies are largely based in protein separation (gel-based techniques and chromatography) and mass spectrometry (MS) for high throughput analysis of proteins in tissues and fluids[8,9]. Quantification methods include isobaric tags for relative and absolute quantification, isotope coded affinity tag and differential image gel electrophoresis, with tandem MS[8,9]. Modifications can also be detected by MS through the corresponding change in mass brought on by the modifying process[5].

Metabolomics involves the study of all the low molecular weight metabolites within a sample that gives a comprehensive reflection of the sample's phenotype at a given time[10]. Identification and quantification of these metabolites can indicate the metabolic processes occurring and changes in these can then be associated with disease processes. Like proteomics, metabolomics is largely driven by MS and chromatography, however there is added complexity due to the variation of the physical properties of these metabolites. Due to this, the metabolites need to be stratified along these properties followed by the application of analytic methods optimised for each type of metabolite[10].

MOLECULAR SUBTYPES FOR PERSONALISED MEDICINE

Genomic subtypes

Waddell *et al*[11] utilised WGS to map the genome of 100 PDAC specimens. Their findings reinforced the known main drivers of PDAC (KRAS, TP53, CDKN2A and

SMAD4) and also found multiple other mutations at much lower prevalence. The authors described four subtypes of PDAC based on the quantity of variations in chromosome structure: (1) Stable (20%; < 50 structural variations and widespread aneuploidy); (2) Locally rearranged (30%; significant events on 1-2 chromosomes); (3) Scattered (36%; < 200 structural variations and moderate chromosomal damage); and (4) Unstable (14%; large number of structural variation and defects in DNA damage repair). The unstable subtype was associated with a high BReast CAncer gene (BRCA) mutational signature, suggesting defects in DNA damage repair, possibly sensitising these to platinum based chemotherapy or poly (ADP-ribose) polymerase inhibitors (PARPi). Indeed, from their sample, 4 out of 5 of these patients treated with platinum based chemotherapy showed response to treatment[11].

Singhi *et al*[12] utilised real time targeted sequencing of exons and introns of 3594 PDAC specimens during the course of clinical care and reported that 17% of these may be susceptible to current therapies based on this genomic data. They found genetic alterations in receptor tyrosine kinase (RTK)/Ras/mitogen-activated protein kinase (MAPK) activation, DNA damage repair, cell cycle control, TGF-B signalling, histone modification, SWI/SNF protein complex, PI3L/mTOR signalling, WNT/B-catenin pathway, RNA splicing, NOTCH pathway, angiogenesis and Hedgehog signalling. Interestingly, the investigators also found that 14% of their sample exhibited mutations in DNA damage repair genes (BRCA-FANC family). Further to this, they identified genetic alterations in receptor tyrosine kinases as potential targets on a background of wild-type KRAS PDAC (12% of the sample)[12].

Aguirre *et al*[13] performed deep WES of PDAC primaries and metastases for 73 patients having clinically indicated biopsies. Average time for the results of WES to return to the clinicians was 39 d, longer than that of research only biopsies (28 d) due to the need for histological diagnosis prior to sequencing. Analysis of these findings resulted in three mutational signatures: SigA (homologous recombination deficiency), SigB (aging) and SigC (unknown aetiology). Around 40% of these patients had potentially targetable genomic findings, when excluding KRAS or CDKN2A, 48% were eligible for clinical trials or off-label use of other therapeutic agents and 24% of patients were indeed enrolled onto a clinical trial or treated with an off-label agent. This shows the feasibility of implementing WES clinically to guide treatment choice. Clinically relevant findings included DNA damage repair (DDR) mutations and BRAF mutations in KRAS wild-type PDAC which may confer sensitivity to platinum-based chemotherapy/PARPi and MAPK inhibition respectively. The authors also presented two such case studies with significant responses to treatment[13].

Proteomic subtypes

Using 56 PDAC liver metastases specimens, Law *et al*[14] performed liquid chromatography-mass spectrometry (LC-MS/MS) to identify 30811 peptides that mapped to 916 proteins comprising of at least 5 peptides in 80% of the sample. Functional analysis of these proteins showed that they play a role in, "extracellular matrix organization, protein processing and transport, translation, glycolytic processes, NADPH metabolism, cell migration, immune response, fibronectin binding, and cell homeostasis"[14].

These proteins were analysed to categorise four PDAC subtypes and three protein clusters. The matched subtypes and clusters are: "Inflammatory" (cluster 3 - pentose phosphate pathway, adaptive immune response, complement activation, IL8 production and extracellular fibril organisation), "proliferative" (cluster 2 - translation, cell proliferation and telomere maintenance), "progenitor-like" (cluster 1 - ethanol oxidation pathways, mitochondrial fatty acid B-oxidation and retinoic acid signaling pathways) and "metabolic" (cluster 1). Both the "progenitor-like" and "metabolic" subtypes exhibit cluster 1 proteins however, the latter shows higher expression of these proteins. The authors were also able to map these subtypes onto previously defined transcriptomic subtypes, making this the first proteomic study with data robust enough to make such a correlation[14].

Clinically, the "proliferative" subtype was associated with a history of alcohol use and the "metabolic" subtype was associated with tobacco use. The "metabolic" and "progenitor-like" subtypes had a decreased risk of death when treated with FOLFIRINOX + gemcitabine compared to the other two subtypes. Further analysis also showed that there was a significant increase in survival when "progenitor-like" subtypes are treated with gemcitabine and that the "metabolic" subtype had a negative correlation between survival probability and abraxane/paclitaxel treatment. These analyses support the use of proteomics derived subtypes as a method for selecting targeted therapeutics however more robust trials are needed to validate these findings [14].

Another interesting finding from this study is the role of serine hydro- xymethyltransferase (SHMT1) (involved in the folic acid cycle) in gemcitabine resistance. Comparison of untreated samples and samples treated with only gemcitabine showed that SHMT1 was significantly down-regulated in the treatment group. The investigators further displayed increased EC50 of gemcitabine in cell lines with SHMT1 knockdown compared to the control group, showing that SHMT1 is a potential mediator of gemcitabine resistance. Expression of SHMT1 was higher in "metabolic" and "progenitor-like" subtypes compared to the other two subtypes regardless of gemcitabine treatment. Expression of this protein may potentially guide the choice of gemcitabine as treatment or monitor those on gemcitabine for resistance to treatment [14].

Humphrey *et al*[15] used liquid chromatography-mass spectrometry (LC-MS/MS) to stratify two cohorts of PDAC cell lines American Type Culture Collection (ATCC) and The Kinghorn Cancer Centre (TKCC), along tyrosine phosphorylation (pTyr) sites. The authors produced a list of 1622 pTyr sites from 797 proteins. Of these, 144 had significant subtype specificity. ATCC subtype 1 showed hypophosphorylation of 65 pTyr sites and the enriched proteins were involved in formation and regulation cell-cell adheren junctions and tight junctions. ATCC subtype 2 contained 54 up-regulated pTyr sites (specifically increased relative phosphorylation rather than increased protein levels) with enrichment of proteins involved in mRNA processing and spliceosome pathways. ATCC subtype 3 showed significantly increased phosphorylation (in both relative phosphorylation and protein expression) in 15 pTyr sites of RTKs including EGFR, MET, RON, EPHA4, EPHB2/3/4 and DDR2[15].

When this methodology was applied to the TKCC cohorts, 1220 pTyr sites were identified, of which 383 were subtype specific. TKCC subtypes 1 and 2 showed 101 and 73 down-regulated pTyr sites, while TKCC subtype 3 showed up-regulation of 209 pTyr sites and was enriched for Ephrin and EGFR signalling. Targeting RTK pTyr sites in the TKCC cohort showed increased phosphorylation of RTKs in subtype 3 including sites on EGFR, EPHA2, DDR1, FGFR1, INSR, MERTK, MET, and RON[15].

Of the subtype specific pTyr sites identified, 8 were identified as "common classifier sites", able to predict the subtype in the ATCC cohort. Subtypes 1, 2 and 3 were identified to exhibit low medium and high phosphorylation of these sites respectively. As both cohorts had subtypes that were "RTK-enriched", the investigators tested the cell lines in this cohort against erlotinib, an EGFR kinase inhibitor. Indeed, the cell lines in this subtype showed increased sensitivity to erlotinib. The authors recognised that the pTyr signature of these RTKs are what conferred sensitivity to RTK blockade rather than expression levels and so stratifying patients with such a signature can potentially allow targeted therapeutic regimes to be studied[15].

Metabolomic subtypes

Metabolomics profiling by Daemen *et al*[16] using LC-MS/MS and gas chromatography-MS represents the only metabolomic study to stratify PDAC. The investigators examined 38 "PDAC-derived" cell lines to quantify 256 metabolites. Analysis of these metabolites revealed three subtypes of PDAC, described as: (1) Slow proliferating (34%); (2) Glycolytic (27%); and (3) Lipogenic (39%). The slow proliferating subtype was low in amino acids and carbohydrates, and the cells had a significantly longer doubling time than the other two subtypes. The glycolytic subtype showed increased levels of metabolites of the glycolytic and serine pathways (phosphoenolpyruvate, glyceraldehyde-3-phosphate, lactate, and serine) and decreased redox balance metabolites (NAD, NADH, NADP, NADPH, GSSG, GSH and flavin adenine dinucleotide). The lipogenic pathway showed increased levels of lipid metabolites (palmitic acid, oleic acid, palmitloeic acid and myristic acid), oxidative phosphorylation (OXPHOS) metabolites (coenzymes Q9 and Q10) and aspartate-malate shuttle metabolites (aspartate and glutamate)[16].

Moreover, the authors predicted and demonstrated in vitro, sensitivity of the glycolytic subtypes to aerobic glycolysis inhibitors (oxamate and the LDHA inhibitor GNE-140), glutaminolysis inhibitor (BPTES) and inhibitors of gamma-glutamylcysteine synthetase (BSO) and cystine transporter [(S)-4-CPG], and also demonstrated sensitivity of the lipogenic subtype to lipid synthesis inhibitors (FASN inhibitor GSK1195010, SCD inhibitor, cerulenin, and orlistat). They also performed *in vivo* confirmatory tests, inducing 68% tumour growth inhibition of the glycolytic cell line with LDHA inhibition and 52% tumour growth inhibition in the lipogenic cell line with SCD inhibition[16]. Table 1 summarises these studies and the clinically significant findings.

Table 1 Summary of molecular subtyping studies

Ref.	Subtypes	Clinical significance
	Genomic	
Waddell *et al* [11]	Stable, locally re-arranged, scattered and unstable	High BRCA mutational signature in the unstable subtype, sensitizing to PARPi and PBC.
Singhi *et al*[12]	-	Real time genetic sequencing. 17% of specimens found to have sensitivities to available treatments. Potential therapeutic targets.
Aguirre *et al* [13]	SigA, SigB and SigC	Potential targets in 40% of patients. 48% eligible for trials/off-label use. Of 24% enrolled onto a clinical trial.
	Proteomic	
Law *et al*[14]	Inflammatory, proliferative, progenitor-like and metabolic	↓Risk of death in metabolic and progenitor-like subtypes treated with FOLFIRINOX+Gemcitabine. ↑Survival in progenitor-like subtype treated with gemcitabine. SHMT1 a potential mediator of gemcitabine resistance.
Humphrey *et al*[15]	TKCC subtypes 1, 2 and 3 ATCC subtypes 1, 2 and 3	Subtypes 3 in both cohorts showed increased sensitivity to erlotinib, potentially mediated by tyrosine phosphorylation of RTK sites.
	Metabolomic	
Daemen *et al* [16]	Slow proliferating, glycolytic and lipogenic	Glycolytic subtype sensitive to inhibitors of aerobic glycolysis, glutaminolysis, γ-glutamylcysteine and Xct. Lipogenic subtype sensitive to lipid synthesis inhibitors.

BRCA; Breast cancer gene; RTK: Receptor tyrosine kinase; PBC: Platinum-based chemotherapy; PARPi: Poly (ADP-ribose) polymerase inhibitors; xCT: Cystine transporter; SHMT1: Serine hydroxymethyltransferase 1.

BIOMARKER DISCOVERY

Currently, the only biomarker for PDAC is carbohydrate antigen 19-9 (CA19-9), approved by the Food and Drug Administration for use in clinical practice[17]. Unfortunately, the median sensitivity and specificity of CA19-9 is 79% and 82% respectively, making it unsuitable for use as a diagnostic marker, with it being raised in other gastrointestinal pathology[18]. Further complicating this is the fact that roughly 10% of the population with a Lewis-negative genotype do not express CA19-9 (a sialyl-Lewis A tetrasaccheride) at all. Its use currently is limited to monitoring CA19-9 positive PDAC for progression or recurrence after resection[17]. There is an obvious need for further investigation for potential biomarkers for early diagnosis, prognosis and sensitivity/resistance to therapeutics. "Omics" techniques have the capability to produce large amounts of data which can be correlated with specific states of the biological system and so there is great potential for this data to be used for biomarker discovery.

As mentioned earlier, one of the main factors that influence the outcomes in PDAC is that the majority are diagnosed at an advanced stage. With surgical resection currently being the only curative option, improving the proportion of patients eligible for surgery would drastically improve outcomes. The use of "omics" technologies to screen for potential diagnostic markers that can accurately differentiate PDAC from other pathologies or normal healthy tissue can lead the way to targeted diagnostic panels that can be implemented clinically.

An emerging area of research is the use of "liquid biopsy", which is the sampling of tumour material which spills into the circulation[19]. The main components of such a biopsy include circulating tumour cells (CTC), cell-free DNA (cfDNA) and circulating exosomes. cfDNA is genetic material released into the circulation from benign and malignant cells during cell death, circulating tumour DNA (ctDNA) being the subset of this derived from malignant cells. Exosomes are extracellular vesicles released from cells into various bodily fluids and can contain proteins and genetic material for analysis. They have a longer half-life and are constantly being produced by cells making them more readily available for isolation than cfDNA[19].

Zhu *et al*[20] conducted a systematic review and meta-analysis of 19 studies utilising ctDNA, CTCs and exosomes to diagnose "pancreatic adenocarcinoma", "pancreatic ductal adenocarcinoma" or "pancreatic cancer". They found that the overall sensitivity, specificity and area under the curve (AUC) for liquid biopsy was 0.8 [95% confidence interval (CI): 0.77-0.82], 0.89 (95%CI: 0.87-0.91) and 0.93 respectively, demonstrating the feasibility of liquid biopsy as a diagnostic tool. Of the three components,

exosomes were found to have the highest accuracy with a sensitivity, specificity and AUC of 0.93 (95%CI: 0.90-0.95), 0.92 (95%CI: 0.88-0.95), and 0.9819 respectively. The authors suggested that the high sensitivity of the exosomes was due to the exocrine function of the pancreases and the high specificity due to the methods used to analyse the exosomes in these studies [polymerase chain reaction (PCR) and flow cytometry] [20].

Overall sensitivity, specificity and AUC of the CTC studies was 0.74 (95%CI: 0.68-0.79), 0.83 (95%CI: 0.78-0.88), and 0.8166[20]. This was thought to be due to hepatic trapping of CTCs and due to reduced blood flow in pancreatic malignancies compared to normal tissue (decreasing the chances of cell shedding into the circulation)[20]. Indeed, it was found that for PC, the levels of CTC was the lowest compared to other types of cancer[21].

ctDNA's overall sensitivity, specificity and AUC was 0.64 (95%CI: 0.58-0.70), 0.92 (95%CI: 0.88-0.95), and 0.9478[20]. All of the ctDNA studies utilised PCR of KRAS mutations to distinguish PC from either healthy controls, pancreatitis or benign lesions. As mentioned before, KRAS is the most common genetic driver of PDAC and so utilising this as the marker for detection may be the reason for the high specificity. The relatively low sensitivity however may have been due to the abundance of ctDNA in the circulation[20]. It has been found that the abundance of ctDNA has a positive correlation with tumour load, supported by the physiology of ctDNA mentioned above: It is released through cell death[22]. Due to this, ctDNA may not be an ideal candidate for use in early detection of PDAC but may have a role as a prognostic biomarker or a marker of response to treatment, especially in those that are CA19-9 negative.

In terms of protein biomarkers, many studies have been done to characterise the differences in the proteomes of PDAC and normal control (NC) specimens from various sources including tissue samples, cell lines, serum/plasma and pancreatic juice[8]. While quite a few studies have been done, only a few of the biomarkers described overlap between studies. Further to this, none of these biomarkers have been put to use clinically, mainly due to the lack of validation in clinical trials, and standardised, reproducible and cost-effective analytical methods.

MS data combined with the results of a literature review by Capello *et al*[23] yielded 17 plasma protein biomarkers to distinguish early stage PDAC from benign pancreatic disease and NC. They tested these biomarkers using ELISA, first in a triage set which narrowed these down to 7 biomarkers, followed by validation of these 7 biomarkers in three independent plasma sample sets. Statistical analysis of the performance of these 7 biomarkers led to the development of a 3 biomarker panel of metalloproteinase inhibitor 1, leucine rich alpha-2-glycoprotein 1 and CA 19-9 which was able to differentiate PDAC cases from healthy controls with an AUC of 0.887 (95%CI: 0.817-0.957) in a blinded test set, which was a statistically significant improvement compared to CA 19-9 alone. The sensitivities at a fixed 95% and 99% specificity were 0.667 and 0.410 respectively, compared to the sensitivities of 0.538 and 0.462 respectively for CA 19-9 [23].

In terms of prognostic biomarkers, de Oliveira *et al*[24] recently conducted a meta-analysis of MS data from two systematic reviews of PDAC secretome and proteome. No protein was found to be present in all the studies and so the authors selected those that were presented in at least 2 studies, generating a list of 39 secreted proteins. Further gene expression analysis of 4747 tumours (of 10 types of cancers) and 2737 corresponding normal tissues for these proteins revealed that 31 of these were unregulated in PDAC, and when analysed for 10 cancer types showed that all 39 genes were enriched in PDAC *vs* the other types, with an opposite expression profile in acute myeloid leukaemia. Further to this, the authors displayed a correlation between the gene expression for these proteins and significantly shorter survival (hazard ratio = 5.36) in PDAC patients, which was validated in three independent data sets[24].

Peng *et al*[25] developed a protein signature to predict response to chemotherapy through LC-MS/MS of prepared serum samples from 16 stage IV PDAC patients. The three protein biomarker candidates vitamin-K dependent protein Z, sex hormone-binding globulin and von Willebrand factor, combined with CA 19-9 were used to make a biomarker panel, with biomarker positive patients having significantly shorter median survival in both stage III and stage IV patients [8.7 mo (95%CI: 6-11.7) for biomarker negtive *vs* 19.2 mo (95%CI: 11.4-22.1) for biomarker positive].

A meta-analysis of metabolomic biomarkers for PC by Mehta *et al*[26] yielded 21 deregulated blood based biomarkers that appeared in at least 2 studies. The authors developed a 10 metabolite diagnostic panel from these biomarkers which was tested on plasma samples of 192 patients from four diagnostic groups: PC (n = 59), NC (n = 48), colorectal cancer (CRC, n = 66) and type 2 diabetes mellitus (T2DM, n = 19). The

Table 2 Summary of biomarker discovery studies

Ref.	Biomarkers	Sensitivity	Specificity	AUC
Zhu *et al*[20]	ctDNA	0.64	0.92	0.9478
	CTC	0.74	0.83	0.8166
	Exosome	0.93	0.92	0.9819
Capello *et al*[23]	TIMP1, LRG1 and CA19-9	0.667/0.410	0.95/0.99	0.887
Mehta *et al*[26]	Panel of: Lactate, LysoPC (18:2), Alanine, Choline, Threonine, Asparagine, Tyrosine, Lysine, Palmitate and 3-hydroxybutyrate	-	-	0.992 *vs* NC
				0.957 *vs* T2DM
				0.653 *vs* CRC
		Biomarker + ve median survival		Biomarker - ve median survival
Peng *et al*[25]	Panel of: PZ, SHBG, VWF and CA19-9	19.2 mo		8.7 mo
			AUC	
Martín-Blázquez *et al*[27]	Panel of: PS (12:0/15:1), TG (22:2/15:0/18:3), 4-oxo-Retinoic acid, Androsterone sulfate, LysoPE (18:2), Phenylalanylphenylalanine, all-trans-Decaprenyldiphosphate, LysoPC (18:2) and Dehydroepiandrosterone sulfate		0.992 (CI: 0.972-1.000)	

ctDNA: Circulating tumour DNA; CTC: Circulating tumour cells; TIMP1: Metalloproteinase inhibitor 1; LRG1: Leucine rich alpha-2-glycoprotein 1; CA19-9: Carbohydrate antigen 19-9; CI: Confidence interval; NC: Normal control; T2DM: Type 2 Diabetes mellitus; CRC: Colorectal cancer.

AUC of PC, T2DM and CRC *vs* NC were 0.992 (95%CI: 0.977-1), 0.957 (95%CI: 0.868-1) and 0.986 (95%CI: 0.967-1) respectively. The AUC of PC *vs* CRC was only 0.653 (95%CI: 0.543-0.757), suggesting a lack of specificity of the panel between these two groups. An index of the 10 metabolite panel showed that higher index values correlated with increased risk of malignancy, with a value of ≥ 12.5 representing a 100% risk of PC [26]. While the authors used the term "pancreatic cancer", in their study, the authors manually curated the literature to ensure the comparison groups were PDAC patients and controls.

More recently, Martín-Blázquez *et al*[27] analysed serum from unresectable PDAC patients and NC using reverse-phase liquid chromatography and high-resolution MS to identify 86 significant metabolites. With these, the researchers proposed a model of 9 markers that discriminated PDAC from healthy controls with an AUC of 0.992 (95%CI: 0.972-1.000). Table 2 summarises these molecular subtyping studies while Figure 1 demonstrates subtypes and biomarkers by "Omics" level[28].

CONCLUSION

Closing thoughts and future considerations

"Omics" technologies have allowed mining of massive amounts of data, giving new insights into the complex, heterogeneous nature of PDAC. As described above, many studies have been done to describe molecular classifications and potential biomarkers, but none of these have yet been translated into clinical practice[28]. Several trials, as reviewed by Du *et al*[29], faced difficulties in sample procurement, low quality of samples and waiting time for sample analysis leading to patient deterioration or withdrawal. The authors suggest a multi-disciplinary approach with specialist input in sample acquisition in designated centres with high levels of experience. Issues regarding the technology should improve as these become more accessible to the clinical setting. Indeed, these difficulties were echoed by lessons learnt from the Individualized Molecular Pancreatic Cancer Therapy trial, a study that aimed to match patients with recurrent or metastatic PDAC to treatment based on genomic data[30]. Unfortunately, no patients were able to be enrolled into the study largely due to the quick progression of PDAC and due to inadequate numbers meeting the eligibility criteria. The investigators described four criteria that would need to be met for such a study to take place: (1) Screening of a sufficient number of patients; (2) Timely acquisition of tumour material for analysis; (3) Quick turnaround time of usable data; and (4)

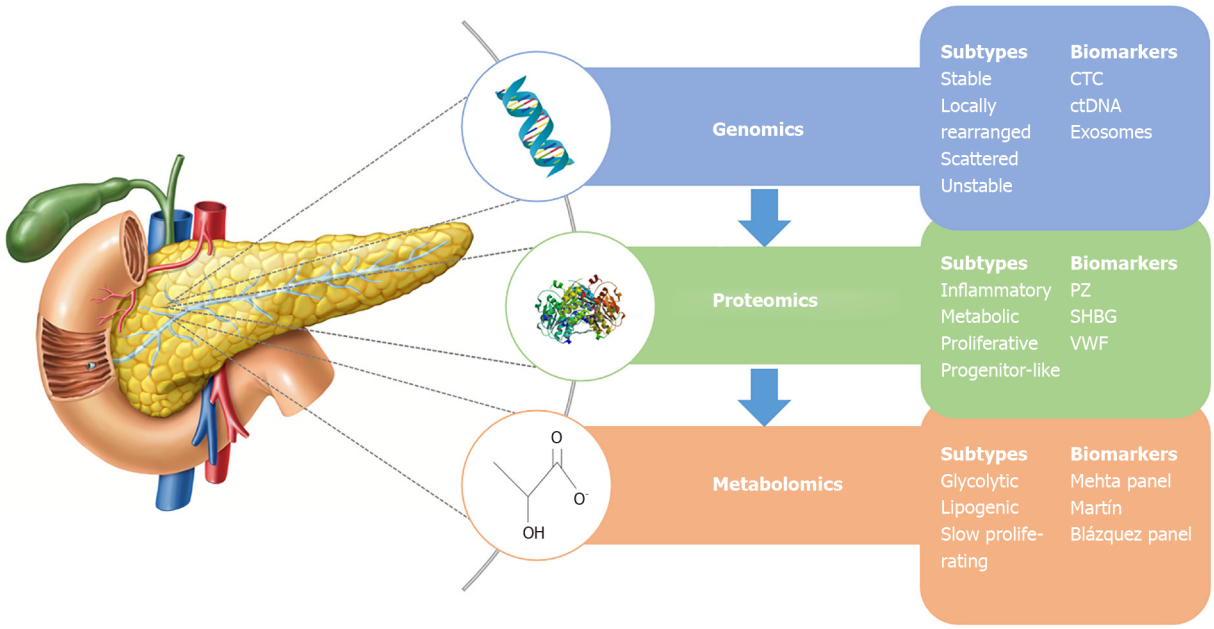


Figure 1 Genomic, proteomic and metabolomic subtypes and biomarkers of pancreatic cancer. Double stranded DNA image[28]. CTC: Circulating tumour cells; ctDNA: Circulating tumour DNA; PZ: Vitamin-K dependent protein Z; SHBG: Sex hormone-binding globulin; VWF: Von Willebrand Factor.

Effective treatments or clinical trials for enrollment[30]. Currently, there is an exciting initiative by PrecisionPanc in the United Kingdom, where through a "Master Protocol", patients suspected or diagnosed with PDAC will undergo prospective molecular profiling to guide enrollment into one of their five PRIMUS trials[31].

Additionally, many of these studies are largely comparative, most of them describing the difference in data between disease and normal states. Few studies have correlated their findings with upstream/downstream "omics" data and so there is an obvious need for integrated analysis of multiple "omics" levels. An example of integrated analysis is seen in the study by The Cancer Genome Atlas Research Network, who performed genomic, transcriptomic and proteomic analysis of 150 PDAC specimens using whole-exon sequencing, DNA methylation assays, RNA sequencing and reverse phase protein arrays[32]. The authors described molecular profiles along each of these "omic" levels, potential subtypes along transcriptomic and proteomic lines, potential therapeutic targets and through integrated analysis were able to describe some of the interplay between these levels, giving further insight into the complexity of PDAC. Another example is the study by Follia *et al*[33] who used genomic and transcriptomic data to describe four metabolic subtypes of PDAC (2 glycolytic and 2 non-glycolytic) which they correlated with the subtypes described by Daemen *et al*[16].

Finally, with such high throughput technologies being used, cancer research is moving into the realm of "big data". "Wide" data sets (where the number of variables exceed the number of subjects) such as those produced by "omics" technologies are better analysed through machine learning/artificial intelligence than traditional statistical analysis[34]. Over the past few years, many studies have been done using machine learning methods for molecular subtyping and biomarker discovery in other types of malignancies, simultaneously using data from multiple "omics" levels and there is great potential for machine learning and artificial intelligence applications in PDAC research[35].

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MINIREVIEWS

Screening and prevention of hepatitis C virus reactivation during chemotherapy

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Abstract

Hepatitis C virus (HCV) reactivation occurs in 23% of HCV-infected cancer patients receiving chemotherapy. Forty-three percent of the patients with reactivation of HCV during chemotherapy develop a hepatitis flare. Most of the cancer patients with HCV reactivation have an unremarkable clinical course following an HCV-related hepatitis flare during chemotherapy. However, 26%–57% of the cancer patients developing an acute flare of chronic hepatitis C during chemotherapy require unanticipated discontinuation or dose reduction of chemotherapy, which results in deleterious changes in the cancer treatment plan. Although an optimal strategy for HCV screening in cancer patients receiving chemotherapy has not been established, universal pre-chemotherapy HCV testing for patients with hematological malignancies is recommended by current guidelines. All the currently approved direct-acting antivirals (DAAs) can be used in cancer patients, but the use of DAAs during chemotherapy should avoid drug–drug interactions between chemotherapy and antiviral agents. If there are no contraindications or anticipated drug–drug interactions, DAAs treatment can be administered before, during, or after chemotherapy. In conclusion, HCV reactivation occurs in approximately one-fourth of HCV-infected cancer patients receiving chemotherapy. An HCV-related hepatitis flare during chemotherapy may lead to the discontinuation of potentially life-saving chemotherapy.

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Currently, universal HCV screening is recommended in hematological malignancy patients before chemotherapy, but there is no evidence-based guideline for other cancer patients. DAAs treatment can cure HCV infection and prevent HCV reactivation during chemotherapy.

Key Words: Hepatitis C virus; Chemotherapy; Screening; Reactivation; Hepatitis flare

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Core Tip: Hepatitis C virus (HCV) reactivation occurs in approximately one-fourth of HCV-infected cancer patients receiving chemotherapy. An HCV-related hepatitis flare during chemotherapy may lead to the discontinuation of potentially life-saving chemotherapy. Currently, universal HCV screening is recommended in hematological malignancy patients before chemotherapy, but there is no evidence-based guideline for other cancer patients. direct-acting antivirals treatment can cure HCV infection and prevent HCV reactivation during chemotherapy.

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INTRODUCTION

Hepatitis C virus (HCV) infection affects millions of people worldwide and is a significant burden for cancer patients[1,2]. The prevalence of chronic HCV infection among cancer patients in the United States ranges from 1.5% to 10.6%[3]. A recent study with universal pre-chemotherapy screening of HCV infection by testing for anti-HCV antibody showed that the prevalence of HCV infection in cancer patients receiving chemotherapy was 6.0% (337/5601) in Taiwan[4]. Chemotherapy can lead to immunosuppression and reactivate quiescent HCV infection in cancer patients[5-13]. The acute flare of HCV infection may result in deleterious changes in the cancer treatment plan and has a negative impact on the treatment outcome of cancer patients. Currently, evidence-based guidelines for HCV screening and treatment in cancer patients undergoing chemotherapy have not been established because data on the efficacy of pre-chemotherapy HCV testing and treatment are very limited. In this article, we review the incidence of HCV reactivation during chemotherapy and the outcome of HCV-related hepatitis flare in cancer patients receiving chemotherapy. Additionally, the recommendations for the pre-chemotherapy HCV screening and the prevention of HCV reactivation during chemotherapy are also reviewed.

DEFINITION OF QUIESCENT HCV INFECTION, HCV REACTIVATION AND HCV-RELATED HEPATITIS FLARE IN CANCER PATIENTS RECEIVING CHEMOTHERAPY

This article summarizes current evidences dealing with HCV reactivation and HCV-related hepatitis flare in cancer patients receiving chemotherapy. It is worthy to note that the definitions of HCV reactivation and hepatitis flare during chemotherapy varied in previous studies[4,14-22]. In this review article, we list the definitions of HCV reactivation and HCV-related hepatitis flare in each study quoted in the Tables. The definitions of HCV reactivation and HCV-related hepatitis flare recommended by authors are summarized in Table 1. Recommended HCV reactivation during chemotherapy is increase in HCV-RNA level of ≥ 1 Log_{10} IU/mL over baseline[17], and recommended definition of HCV-related hepatitis flare is unexplained increase in alanine aminotransferase (ALT) to 3 times the upper limit of normal during chemotherapy and increase in HCV-RNA level of ≥ 1 Log_{10} IU/mL over baseline[18]. Most retrospective works lacked the data of HCV viral load before chemotherapy. The

Table 1 Recommended definitions of quiescent hepatitis C virus infection, hepatitis C virus reactivation and hepatitis C virus-related hepatitis flare in cancer patients receiving chemotherapy

Term	Definition	Ref.
Quiescent HCV infection	An HCV infection with a positive serum HCV-RNA and normal serum levels of liver enzymes	IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, No. 59[30]
HCV reactivation	Increase in HCV-RNA level during chemotherapy of ≥ 1 $\log_{10}$ IU/mL over baseline	Talima *et al*[17]
HCV-related hepatitis flare	Unexplained increase in ALT to 3 times the upper limit of normal during chemotherapy and increase in HCV-RNA level of ≥ 1 $\log_{10}$ IU/mL over baseline	Torres *et al*[18]

IARC: International Agency for Research on Cancer; HCV: Hepatitis C virus.

scientific strengths of these retrospective evidences were therefore not robust.

INCIDENCE OF HCV REACTIVATION IN CANCER PATIENTS RECEIVING CHEMOTHERAPY

In a retrospective observation study by Lee *et al*[16], enhanced replication of HCV (increase in HCV-RNA level of ≥ 1 Log_{10} IU/mL over baseline) was noted in 9 (27%) of 33 HCV-infected cancer patients who underwent chemotherapy. Another retrospective study by Talima *et al*[17] demonstrated that the incidence of HCV reactivation (increase in HCV-RNA level of ≥ 1 Log_{10} IU/mL over baseline) in 34 HCV-infected breast cancer patients receiving chemotherapy was 6% (2/34). In a prospective observation study by Torres *et al*[18], reactivation of HCV infection (increase in HCV-RNA level of ≥ 1 Log_{10} IU/mL over baseline) occurred in 23 (23%) of 100 cancer patients undergoing chemotherapy. Among these cancer patients, those with hematological malignancies had a higher incidence of HCV reactivation than those with solid tumors (36% *vs* 10%).

INCIDENCE OF HCV-RELATED HEPATITIS FLARE IN CANCER PATIENTS RECEIVING CHEMOTHERAPY

HCV-related hepatitis flare is a significant burden for cancer patients undergoing chemotherapy[6,19-20]. Table 2 displays the incidence of HCV-related hepatitis flare in cancer patients receiving chemotherapy. In a retrospective observation study by Li *et al*[4], universal pre-chemotherapy screening of HCV infection by testing for anti-HCV antibody was conducted in 5601 cancer patients undergoing chemotherapy. HCV-infected cancer patients had a higher incidence of severe acute liver injury (serum ALT increases beyond 10 times the upper limit of normal during chemotherapy or 6 mo following chemotherapy) than those without HCV infection (2.3% *vs* 0.7%). Among the HCV-infected patients who did not have chronic HBV infection, the incidences of severe liver injury in those with hematological malignancy, hepatocellular carcinoma (HCC) and non-HCC solid tumors were 9.4% (3/32), 1.9% (2/105), and 1.2% (2/169), respectively. In this study, the incidence of severe acute liver injury in HCV-infected hematological cancer patients was higher than that in those with HCC patients and non-HCC solid tumor patients (9.4% *vs* 1.9 % and 1.1%, respectively). Rituximab-containing chemotherapy and hematological malignancy were identified risk factors related to severe acute exacerbation of HCV infection in cancer patients undergoing chemotherapy. In another retrospective study by Mahale *et al*[5], the incidence of acute exacerbation of HCV infection (3-fold or greater increase in serum ALT level) during chemotherapy was 23% in 104 patients with hematological malignancy and 4% in 204 patients with solid tumors. The former also had a higher incidence of HCV-related acute exacerbation than the latter. In a prospective observation study at MD Anderson Cancer Center[18], reactivation of HCV infection occurred in 23% of 100 cancer patients receiving chemotherapy. Among those with HCV reactivation, 10 patients (43%) developed a hepatitis flare (unexplained increase in ALT to 3 times the upper limit of normal). Overall, the incidence of HCV-related hepatitis flare in cancer patients receiving chemotherapy was 10% in this prospective study.

Table 2 Incidence of hepatitis C virus-related hepatitis flare in cancer patients receiving chemotherapy

Ref.	Study type	Cancer type	Results
Torres *et al* [18]	Prospective observation study	Hematological tumor (n = 50)	A hepatitis flare occurred in 10% of HCV-infected cancer patients receiving chemotherapy
		Non-HCC solid tumor (n = 50)	Definition of HCV-related hepatitis flare: unexplained increase in ALT to 3 times the upper limit of normal and increase in HCV-RNA level of ≥ 1 $\log_{10}$ IU/mL over baseline
Li *et al*[4]	Retrospective observation study	Hematological tumor (n = 569); HCC (n = 256); Non-HCC solid tumor (n = 3900)	The incidence of severe acute liver injury in HCV-infected hematological cancer patients was higher than that in those with HCC patients and non-HCC solid tumor patients (9.4% *vs* 1.9 % and 1.1%, respectively). Definition of severe acute liver injury: ALT increased beyond 10 times the upper limit of normal during chemotherapy or 6 months following chemotherapy
Tomizawa *et al*[28]	Retrospective observation study	Colorectal cancer (n = 24)	The incidence of severe acute exacerbation rate in HCV-infected patients was 8%. Definition of severe acute exacerbation: ALT increased beyond 5 times the upper limit of normal
Hsu *et al* [22]	Retrospective observation study	Hematological tumor (n = 104); Solid tumor (n = 204)	The incidence of HCV acute exacerbation was 11% (hematological tumor: 23%; solid tumor: 4.4%). Definition of HCV acute exacerbation: 3-fold or greater increase in serum ALT level

HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase.

CLINICAL OUTCOME OF HCV-RELATED HEPATITIS FLARE DURING CHEMOTHERAPY

Most of the cancer patients with HCV reactivation have an unremarkable clinical course following HCV flare during chemotherapy. In a prospective observation study by Fujii *et al*[6], 6 (28.6%) of 21 Leukemia patients with HCV-related hepatitis flare during chemotherapy developed hepatic decompensation (Table 3). Another prospective observation study by Torres *et al*[18] showed that none of 23 cancer patients (hematological malignancy: n = 18; solid tumor: n = 5) with HCV-related hepatitis flare during chemotherapy developed liver decompensation or mortality. In a retrospective study including 33 hematological cancer patients with HCV-related hepatitis flare, the incidence of hepatic decompensation was also 0%[7]. Although most of the cancer patients with HCV reactivation or hepatitis flare during chemotherapy have a benign clinical course, a significant number of patients with severe flare of HCV infection have to discontinue potentially life-saving chemotherapy. Currently, there are no randomized controlled trials comparing the outcomes of the patients with chemotherapy-related HCV reactivation who stop chemotherapy and who go on cancer treatment. Therefore, whether chemotherapy should be stopped in cancer patients with HCV reactivation remains unclear. Nonetheless, physicians often discontinue chemotherapy in HCV-infected cancer patients who develop severe liver dysfunction during cancer treatment because it is a life-threatening condition and can be induced by either chemotherapeutic drugs or viral reactivation.

In a retrospective study by Li *et al*[4], four of seven patients (57.1%) with HCV-related severe acute liver injury discontinued chemotherapy due to hepatitis flare. In a prospective study by Torress *et al*[18], 6 of 23 HCV-infected patients (26%) with hepatitis flare required unanticipated discontinuation or dose reduction of chemotherapy. Since interruption of chemotherapy in cancer patients would result in deleterious changes in the cancer treatment plan and has a negative impact on patient outcome, the aforementioned findings support the identification and treatment of chronic HCV infection to prevent HCV reactivation and hepatitis flare.

PRE-CHEMOTHERAPY HCV SCREENING IN CANCER PATIENTS

A standard strategy for HCV screening in cancer patients before chemotherapy has not been established. However, a retrospective study by Hosry *et al*[21] demonstrated that early diagnosis of HCV infection with virological cure improved the outcomes of cancer and survival of HCV-infected patients who developed non-Hodgkin lymphoma. To improve pre-chemotherapy HBV and HCV testing, Hsu *et al*[22] developed a computerized order entry-based therapeutic control system (e-CONTROL) to notify healthcare providers in a medical center in Taiwan for pre-

Table 3 Clinical outcomes of hepatitis C virus-related hepatitis flare during chemotherapy

Ref.	Study type	Cancer type	Liver decompensation	Interruption of chemotherapy	Mortality due to HCV reactivation
Torres *et al*[18]	Prospective observation study	Hematological tumor (n = 18) Solid tumor (n = 5)	Total: 0%	Total: 26%	Total: 0%
Fujii *et al*[6]	Prospective study	Leukemia (n = 21)	Total: 28.6%	–	–
Li *et al*[4]	Retrospective observation study	Hematological tumor (n = 3) HCC (n = 2) Non-HCC solid tumor (n = 2)	Total: 0% (Hematological tumor: 0%; HCC: 0%; Non-HCC solid tumor: 0%)	Total: 57% (Hematological tumor: 67%; HCC: 50%; solid tumor: 50%)	Total: 0% (Hematological tumor: 0%; HCC: 0%; Non-HCC solid tumor: 0%)
Lee *et al*[16]	Retrospective study	Hematological tumor (n = 14) Solid tumor (n = 11)	Total: 0%	Total: 32%	Total: 0%
Mahale *et al*[5]	Retrospective study	Hematological tumor (n = 24) Solid tumor (n = 9)	Total: 0%	Total: 45%	Total: 0%
Zuckerman *et al*[7]	Retrospective study	Hematological tumor (n = 33)	Total: 0%	–	Total: 0%

HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

chemotherapy HBV and HCV testing. The e-CONTROL system achieved a pre-chemotherapy HCV screening rate of 97.7% (5601/5735). The HCV screening rate in cancer patients receiving chemotherapy is low in the United States. According to an observation study at MD Anderson Cancer Center, the HCV screening rate in cancer patients receiving chemotherapy was only 13.9%[23]. Currently, an optimal strategy for HCV screening in cancer patients receiving chemotherapy has not been established. Figure 1 illustrates current recommendations for testing and treating HCV infection in cancer patients receiving chemotherapy[24,25,29]. The 5th European Conference on Infections in Leukemia recommended that all patients with hematological malignancy be screened for hepatotropic viruses (HBV and HCV) before cancer treatment[24]. The European Association for the Study of the Liver (ESAL) recommends that all patients with malignancy should be screened for HCV infection before cancer treatment due to HCV reactivation possibility after treatment[25].

Anti-HCV antibody is a recommended tool for initial screening of HCV infection in cancer patients receiving chemotherapy because of its cheap and cost-effective advantages. However, the disadvantage of screening HCV infection by serum anti-HCV antibody is a positive result indicating either current or past HCV infection. Therefore, cancer patients with a positive result of anti-HCV antibody should be further tested for serum HCV RNA to confirm current infection status of HCV (Figure 1). In clinical practice, serum HCV RNA is not recommended as a routine screening tool for HCV infection in cancer patients because it is an expensive diagnostic method.

TREATMENT OF HCV INFECTION IN CANCER PATIENTS RECEIVING CHEMOTHERAPY

All the currently approved direct-acting antiviral (DAA) agents can be applied in cancer patients, but the use of DAA treatment during chemotherapy should avoid drug–drug interactions between chemotherapy agents and antivirals (Figure 1). The initiation of DAA treatment in HCV-infected cancer patients undergoing chemotherapy should be individualized and determined by the cancer treatment plan. Contraindications to DAA treatment in HCV-infected cancer patients include (1)

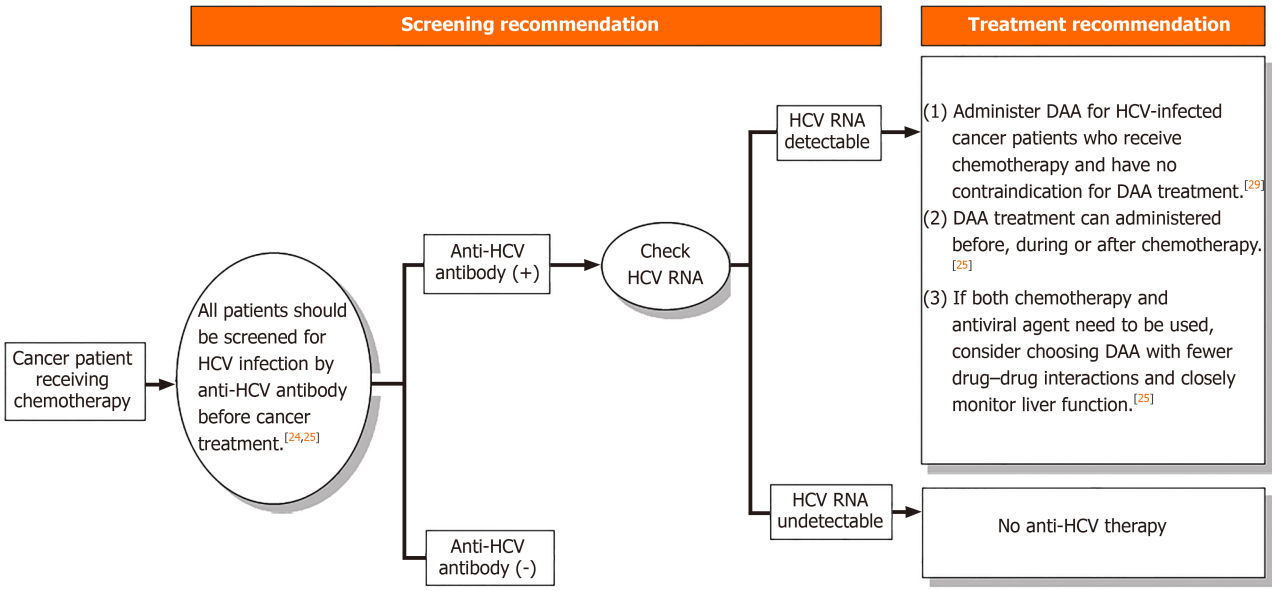


Figure 1 Current recommendations for testing and treating hepatitis C virus infection in cancer patients receiving chemotherapy. HCV: Hepatitis C virus; DAA: Direct-acting antiviral.

Pregnancy; (2) Uncontrolled cancer; (3) Patients with a life expectancy of < 12 mo that cannot be remediated by cancer treatment; (4) Hypersensitivity or intolerance to DAAs; and (5) Anticipated major drug–drug interactions with cancer treatment. If there are no contraindications or anticipated drug–drug interactions, DAA treatment can be administered before, during or after chemotherapy.

The use of DAAs for HCV infection in infected cancer patients receiving chemotherapy may increase the risk of drug–drug interactions. Physicians can identify potentially significant interactions between DAAs and chemotherapy agents based on information obtained from current databases (http://hepdruginteractions.org) and then choose adequate DAAs for HCV treatment or alter the regimen of chemotherapy to avoid drug-drug interactions. In general, treatment for HCV infection in cancer patients receiving chemotherapy by sofosbuvir/Ledipasvir or glecaprevir/pibrentasvir is safe and effective[26]. A prospective observation study from MD Anderson Cancer Center showed that the cure rate of HCV infection by sofosbuvir-based therapy in HCV-infected cancer patients was 91%[27].

CONCLUSION

In conclusion, chemotherapy can lead to immunosuppression and reactivate quiescent HCV infection. Most of the cancer patients with HCV reactivation have an unremarkable clinical course following HCV hepatitis flare during chemotherapy. However, 26%–57% of the cancer patients developing acute exacerbation of chronic hepatitis C during chemotherapy require unanticipated discontinuation or dose reduction of chemotherapy. Currently, the optimal strategy for HCV screening in cancer patients receiving chemotherapy has not been established. Nonetheless, the ESAL recommends that all patients with malignancy should be screened for HCV before cancer treatment due to HCV reactivation possibility after treatment. Currently, universal HCV screening is recommended in hematological malignancy patients before chemotherapy, but there is no evidence-based guideline for other cancer patients. Administration of DAAs can cure HCV infection and prevent HCV reactivation during chemotherapy.

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MINIREVIEWS

Role of near-infrared fluorescence in colorectal surgery

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Abstract

Near-infrared fluorescence (NIRF) is a technique of augmented reality that, when applied in the operating theatre, allows the colorectal surgeon to visualize and assess bowel vascularization, to identify lymph nodes draining a cancer site and to identify ureters. Herein, we review the literature regarding NIRF in colorectal surgery.

Key Words: Fluorescence; Enhanced reality; Anastomotic leak; Ureter; Anastomosis

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Core Tip: Near-infrared fluorescence appears to be a useful tool that assists surgeons performing colorectal surgery by identifying poorly vascularized areas of the bowel and therefore decreasing the incidence of anastomotic leak, visualizing lymphatic drainage and identifying the ureters during difficult surgery.

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INTRODUCTION

Augmented reality (AR) is increasingly used in the operating room to help surgeons in

and hepatology

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Grade D (Fair): 0
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their work. AR is created by overlaying digital information on an image being viewed through a device in real time, allowing us to extend our perception of reality.

Fluorescence is the property of absorbing light of short wavelengths and emitting light of longer wavelengths. In near-infrared (NIR) fluorescence (NIRF) imaging, a fluorescent dye, namely a fluorochrome emitting in the NIR spectrum, is injected intravenously or directly into the tissue. The AR acquisition system functions as a laparoscope but uses two cameras at the same time: A white light camera acquiring real-world images and a second infrared camera for acquiring the fluorescence images emitted by the dye. On the fluorescent images, the rays emitted by the fluorochromes are visualized by white pixels on a black background. The AR image is then constructed by superposition of the real-world image and the recoloured NIR image.

Radiation, like light, penetrates biological tissue while becoming increasingly depleted in energy up to the depth at which all energy transported has been absorbed. Biological tissues absorb and scatter shorter wavelength light, and NIR light can penetrate biological tissues (such as skin and blood) more efficiently than visible light. Therefore, it creates a "transparency" effect of the tissues and allows the acquisition of fluorescent images through several millimetres of depth.

Indocyanine green (ICG) is the most commonly used fluorophore in NIRF imaging and has been approved by the Food and Drug Administration for clinical and research use in humans since 1956. Its fluorescent capabilities have been used increasingly since the 2000s in various specialties, such as neurosurgery, plastic surgery, gynaecology or general surgery. ICG is an iodine dye[1] that can be injected intravenously. The compound is 98% bound to plasma proteins and has a hepatic clearance rate of 18% to 24% *per* minute into the bile. This leads to a half-life of generally 3 to 4 min depending on the vascularization of the organ of interest, with no known metabolites. Its quick clearance rate allows the dye to be used for multiple injections during a procedure. ICG is excited between 750 and 800 nm, and fluorescence is viewed at approximately 830 nm[2]. This fluorochrome is well-tolerated in patients. However, there have been few described cases of hypotension, tachycardia, dyspnoea, urticarial and anaphylactic shock[3]. In colorectal surgery, NIRF imaging uses not only ICG but also other fluorochromes and is employed for three main functions: Estimation of the blood supply, oncological applications and highlighting of anatomical elements such as the ureters.

PREVENTION OF ANASTOMOTIC LEAKS

In colorectal surgery involving intestinal resection, the most feared complication is anastomotic leak (AL), which occurs in 8.1% of procedures according to the European Society of Coloproctology snapshot audit for right hemicolectomies[4] and in up to 17.5% of low colorectal anastomoses. AL causes devastating morbidity and mortality [5,6]. Complete anastomosis healing requires adequate perfusion; therefore, one of the most important risk factors for AL is poor perfusion of the anastomosis[7,8]. Intraoperatively, the selection of an optimal site for anastomosis depends on subjective clinical indicators of good perfusion, such as the colour of the bowel wall, palpable pulsations of the mesenteric arteries, or bleeding of the resection margins[9,10]. Nonetheless, two studies[11,12] showed that the surgeon's subjective AL prediction based on perfusion underestimated the risks of AL. Numerous objective quantitative techniques of intraoperative bowel viability assessment exist, such as measurement of tissue oxygen levels, laser Doppler flowmetry, pulse oximetry, pH measurement and NIR fluorescence angiography[13], but only a few are applicable in colorectal surgery, especially in laparoscopic surgery.

NIR fluorescence angiography using ICG as a fluorochrome is an ever-increasing technique used in the operating room. The first clinical study reporting this method in colorectal surgery was performed by Kudszus *et al*[14] in 2010. ICG was injected intravenously to highlight the microvasculature and enabled surgeons to detect poorly perfused areas more precisely[3]. Indeed, ischaemic demarcation of the intestine is visible with normal light more than 10 min after vessel division, whereas NIRF nearly immediately distinguishes vascularized from ischaemic tissue areas[15]. Fluorescence is visible within 30 to 60 s after fluorochrome injection[16,17], which is performed after the division of the mesenteric vessels when the operator has chosen the resection margins and/or after the anastomosis in order to verify good perfusion of the surrounding tissues.

Thereafter, in a meta-analysis of 10 studies from 2010 to 2017 (894 patients), van den Bos *et al*[18] reported a change in the initial surgical plan in 10.8% of patients. In most articles, the resection was extended to better perfused tissues or the anastomosis was

redone if the perfusion was unsatisfactory[16]. The use of ICG also helped to invalidate the clinical impression of poor perfusion and thus indicated that the resection margins did not need to be extended further. For instance, Kudszus *et al*[14] described a non-extended resection in 2.5% of patients (5/201) despite a clinical impression of poor perfusion. In the study by Kim *et al*[17], the use of ICG determined competent perfusion of the bowel at the anastomosis in 13 patients (10.6%) at risk of ischaemia due to restrictive mesocolon, malrotation, marginal vessel deficiency, accidental left colic artery excision, and oedema due to irradiation.

The effect of NIRF angiography on intraoperative decision-making seems to allow a reduction in the risk of AL (Table 1). However, due to the small sample sizes, only 4 out of 11 studies reported a statistically significant ($P < 0.05$) reduction in AL[16-20]. In other investigations, subgroup analysis showed significant reductions in AL for colorectal cancer (CRC) surgery[14,21], rectal surgery[22], elective resection and hand-sewn anastomosis[14]. However, in two larger studies[23,24], the use of fluorescence angiography did change the decision-making, but there was no discernible change in AL outcome.

The first randomized controlled trials in the field were released in 2019 and 2020 by De Nardi *et al*[25] and Jafari *et al*[26], respectively. In these prospective, single-blinded trials, the authors did not find a significant difference in the AL rate between the NIRF group and the control group (De Nardi *et al*[25]: Incidence of AL = 5% in the NIR group *versus* 9% in the control group; $P = 0.2$; Jafari *et al*[26]: Incidence of AL = 9.0% in the NIR group *vs* 9.6% in the control group; $P = 0.37$). However, these studies might be underpowered. Of note, Jafari *et al*[26] stopped the trial before including the minimum number of 450 patients, as mentioned in their research protocol. Nevertheless, Alekseev *et al*[27] randomized controlled trial including 380 patients showed significant results, with an incidence of AL of 9.1% in the NIR group *vs* 16.3% in the control group ($P = 0.04$). This might be explained by the marked incidence of AL in the included population. Indeed, the authors pro-actively searched for leaks *via* contrast exams performed on every patient 30 d after surgery if the post-operative period was entirely uneventful.

Based on these randomized controlled trials, it can be concluded that the effect of fluorescence probably exists but that this effect is most likely modest and more pronounced in patients with a high risk for AL. New randomized studies are ongoing and will allow us to reach a conclusion on the subject (notably: Armstrong *et al*[28], NCT03602677, NCT03390517).

Regarding economic aspects, AL leads to extended hospitalization. Therefore, the use of NIRs may reduce the length of hospital stay[14] and reduce costs by preventing the occurrence of AL. The initial outlay for the material of the NIR fluorescence system was 70.000€ for Kim *et al*[17], then 13€ *per* patient for the dye; the corresponding cost was 110.000€ (purchase and 5-year maintenance) for Ris *et al*[16], and then 130€ *per* patient for 3 injections. AL increases the treatment costs by €12600[29] to €21500[30]. Therefore, the NIRF technique seems to be cost effective[31], and the acquisition costs of a NIRF-ICG system could be covered in a year if it prevents two ALs *per* year. All the studies do not share this point of view, as Jafari *et al*[32] reported an initial cost of $167500 to $223750 and a cost *per* case of $999 to $1099. Consequently, a randomized controlled trial assessing the effect of NIRF on AL, including a cost-benefit analysis, is needed.

In addition to being used to reduce the rate of AL in elective surgery, NIRF can be used during emergency surgery, *e.g.*, in acute ischaemic disease, to identify bowel segments to be resected. However, only a few studies have applied NIRF to emergency surgery, and these studies included only small numbers of patients ($n = 4$ to 56)[33-35].

In the Liot *et al*[34]'s study, NIR fluorescence led to a change of plan in 32% of the cases: 67% were slated for a less aggressive approach, while only 33% were scheduled for larger resection. None of those patients underwent reoperation for ischaemia. Nevertheless, another study reported two false positive cases where the ICG injection showed good perfusion, while the pathological report finally revealed signs of necrosis.

IDENTIFICATION OF METASTATIC LYMPH NODES

CRC is the third most common cancer worldwide, accounting for approximately 10% of all new cases, and is the fourth most common cause of death from cancer[36]. The 5-year survival of CRC is determined by the stage of the disease at diagnosis and ranges from 90% in early-stage localized tumours to 14% in distant metastatic disease[37].

Table 1 Retrospective cohort studies

Ref.	Indication for resection	Sample size	ICG injection time	Change of plan (%)	AL rate (%)
Kudszus *et al* [14], 2010	Colorectal cancer	ICG: 201. Control: 201	Before anastomosis	16.4	ICG: 3.5. Control: 7.5
Kin *et al*[23], 2015	Colorectal cancer, Diverticulitis, IBD, other	ICG: 173. Control: 173	Before anastomosis	4.6	ICG: 7.5. Control: 6.4
Jafari *et al*[3], 2013	Rectal cancer	ICG: 16. Control: 22	Before anastomosis	19	ICG: 6. Control: 18
Kim *et al*[17], 2016	Rectal cancer	ICG: 123. Control: 313	Before ± after anastomosis	0[2]	ICG: 0.8[a]. Control: 5.4
Boni *et al*[15], 2015	Colorectal cancer	ICG: 42. Control: 38	Before + after Anastomosis	4.7	ICG: 0. Control: 5.2
Wada *et al*[87], 2019	Rectal cancer[1]	ICG: 34. Control: 34	Before anastomosis	27.1[1]	ICG: 8.8. Control: 14.7
Dinallo *et al*[24], 2019	ND	ICG: 234. Control: 320	Before anastomosis	5.6[a]	ICG: 1.3. Control: 1.3
Mizrahi *et al*[88], 2018	Rectal cancer	ICG: 30. Control: 30	Before + after anastomosis	13.3	ICG: 0. Control: 6.7
Kim *et al*[19], 2017	Rectal cancer	ICG: 310. Control: 347	Before anastomosis	ND	ICG: 0.6[a]. Control: 5.2
Ris *et al*[16], 2018	Colorectal cancer (65.5%), diverticular disease (18.8%), Crohn's disease, ulcerative colitis, other	ICG: 504 Control: 1173	Before + after anastomosis	5.8	ICG: 2.4[a]. Control: 5.8
Watanabe *et al* [20], 2020	Rectal cancer	ICG: 211. Control: 211	Before anastomosis	ND	ICG: 4.7[a]. Control: 10.4

[1]149 (48:101).

[2]Fluorescent imaging correctly determined competent perfusion of the bowel adjacent to the anastomosis in 10.6% who were possibly susceptible to anastomotic site ischemia.

[a]$P < 0.05$. ND: No data available; IBD: Inflammatory bowel disease; AL: Anastomotic leak; ICG: Indocyanine green.

Lymph node status is a prognostic factor and a determinant for adjuvant therapeutic intervention. The sentinel lymph node (SLN) concept is based upon the observation that tumour cells migrating from a primary tumour metastasize to one or a few lymph nodes before involving other lymph nodes. This concept was first described in 1960[38], and its application contributed to the identification of lymph nodes with the highest probability of malignant infiltration for the staging of a tumour.

There are various methods for intraoperative mapping of SLNs. Currently, surgeons use direct visualization identification after the injection of dyes such as isosulfan blue [39], patent blue[40], methylene blue[41], or scintigraphy after the injection of radiocolloids around the tumour[42], and for over ten years, the use of NIRF after the injection of ICG.

The capability of ICG NIRF to identify metastatic LNs in various types of malignancies was extensively investigated. The sensitivity of ICG NIRF varied according to the site of the primary malignancy: 50% to 100% for endometrial and cervical carcinoma[43], 50%-100% for gastric cancer[44], and up to 95% to 100% for breast cancer[44]. Lymph nodes and lymph vessels draining the injection site and thus containing ICG can be visualized by NIR without ionizing radiation or radioactivity involvement. In the meta-analysis performed by Emile *et al*[45] in 2017 regarding SLN in CRC, the pooled sensitivity and specificity rates were 71 and 84.6, respectively. On the other hand, when the proportion of patients with early-stage tumours was > 50% of the sample size[46-48], the median sensitivity and specificity increased to 100%. This difference has also been found in the meta-analysis of Burghgraef *et al*[49], where the T1-T2 group had a 1.25 higher accuracy rate than the T3-T4 group (CI: 1.05-1.47). The hypothesis was that the flow through nodes with obvious nodal metastases could be obstructed by the tumour, leading to lymph drainage through alternative pathways (aberrant drainage concept). According to these results, sentinel node mapping with ICG is feasible for early-stage cancer/radiologically localized colorectal neoplasia[50]. Additionally, preoperative T staging should be performed before SLN mapping[46].

The ability of ICG NIRF to detect metastatic LN was compared with patent blue dye in two studies[51,52], which respectively examined both techniques in 20 and in 50 patients with colon cancer and reported that both methods had the same detection rates (95%, 99.5%) for SLN, but higher sensitivity in favour of ICG was observed by Liberale *et al*[51], especially in patients with higher BMIs (> 25 kg/m^2).

Regional lymphadenectomy directly influences the risk of distant failure, provides prognostic information and guides postoperative management, such as chemotherapy administration. The number of nodes retrieved from the surgical specimen improves the accuracy of prognosis and influences survival outcomes in patients with colon cancer. NIR lymph node mapping can modify the surgical procedure when ICG shows additional lymph nodes outside of the proposed resection margins to achieve radical lymph resection for curative surgery[48,53-55]. For that purpose, ICG injection is usually performed between 1 and 3 d before surgery[55]. This technique may serve to optimize and individualize the excision of patient specificities rather than theoretical anatomic generalization.

Another way to use ICG in metastatic lymph node mapping is as an intravenous injection. In tumour tissue, neoangiogenesis is responsible for the presence of immature and permeable vessels allowing ICG-bound molecules to pass into the extravascular space. As the half-life of ICG in blood circulation is approximately 5 min, ICG is rapidly cleared from the vascular space, but extravascular ICG accumulation will be responsible for the observed hyperfluorescence of tumour tissue in contrast to surrounding normal tissue[56]. This phenomenon is known as the enhanced permeability and retention effect. In one study (Liberale *et al*[57], 15 min after intraoperative intravenous injection of ICG, no lymph nodes other than those containing cancer cells were fluorescent, suggesting that fluorescence was directly related to the presence of tumoural tissue inside LNs .

This method also enables us to highlight peritoneal metastasis (PM). The first application of ICG-based fluorescence imaging in patients with PM of colorectal origin was demonstrated in 2016[58]. According to the last study by Lieto *et al*[59], the diagnostic performance of ICG NIRF was significantly better than preoperative and intraoperative conventional procedures. In this study, intraoperative ICG identified additional hyperfluorescent metastatic nodules and then confirmed them by histopathology (16/17). The sensitivity increased from 76.9% to 96.9% with NIRF.

PREVENTION OF IATROGENIC URETERAL INJURY

Iatrogenic ureteral injury is a complication occurring in 0.15% to 1.9%[60,61] of patients undergoing colorectal surgery. These lesions include ureteral sections, ligations, crushing, coagulation or indirect injuries, such as burn and ischaemic lesions, and may require secondary/subsequent surgery in a later stage. Even if it is a rare event, the consequences of such injuries are important and lead to increased postoperative mortality, morbidity, hospital stay and health costs[62].

Preoperative ureter stent placement is a technique used in surgery to help identify the ureters. These stents can be palpated during open surgery, but in laparoscopic surgery, in which tactile feedback is limited, this technique loses its interest[63]. In an effort to enhance visualization of ureters throughout laparoscopic dissection, lighted ureteral stents were devised[64,65]. However, preoperative ureteral stent placement is an invasive procedure that harbours increased risks of complications, such as ureteral perforation and urinary tract infection[66]. New techniques have been or are being developed for ureter visualization with AR assistance (Table 2).

Because of the hepatic clearance of ICG, its use in ureter visualization requires the placement of a ureteral catheter for retrograde injection of the dye directly into the lumen of the ureter. This technique allows identification of the entire course of the ureters from the surrounding tissue in laparoscopic and open surgery and enables the location of a lesion made during the surgery in real time by leakage of the dye in the intraperitoneal space[67]. A small cohort study including 10 patients showed 100% visualization of ureters directly after injection[68]. The importance of this method was confirmed by two case studies[69,70]. Considering the potential complications of ureteral catheters, new dyes were investigated to offer non-invasive alternatives to improve preoperative ureteral identification.

Methylene blue is a dye that has long been used in humans with a good safety profile. Its NIR fluorescent properties were discovered at micromolar concentrations only ten years ago[71]. When used at the typical clinical concentration of 1% (*i.e.*, 31.3 mmol/L), methylene blue is bright blue but has virtually no NIRF because of

Table 2 Ureteral visualization studies using intravenous dye

Ref.	Model	Surgery	Dye	*n* (%)	Visible ureters	Visible time after injection	Visibility duration
Matsui *et al*[71], 2010	Pig	Open[1]	Methylene blue	20	100% (40/40)	10 min	65 min
		Laparoscopy[1]		2	50% (2/4)	10 min	20 min
Verbeek *et al*[89], 2013	Human	Open[1]	Methylene blue	12	100% (20/20)	10 min	≥ 50 min
Al-Taher *et al*[90], 2016	Human	Laparoscopy	Methylene blue	10	62,5% (5/8)	ND	ND
Barnes *et al*[72], 2018	Human	Laparoscopy	Methylene blue	34	93.6% (59/63)	0 min	2 h
		Open		6	66.6% (4/6)		
Tanaka *et al*[67], 2007	Rat	Open	IRDye 800CW	12	100%	3-5 min	ND
	Pig	Open	IRDye 800CW	6	100%	10 min	> 20 min
Schols *et al*[74], 2014	Pig	Laparoscopy	IRDye 800cw	2	50% (1/2)	10 min	ND
Korb *et al*[75], 2015	Pig	Laparoscopy	IRDye 800CW	6	100%	10 min	≥ 50 min
Van den Bos *et al*[18], 2018	Pig	Laparoscopy	IRDye 800 BK	1	100% (2/2)	35 min	3 h
			IRDye 800NOS	1	100% (2/2)	45 min	ND
			IRDye 800CW	1	100% (2/2)	10 min	≥ 15 min
Al-Taher *et al*[77], 2018	Pig	Laparoscopy	IRDye 800BK	3	100% (6/6)	1-20 min	≥ 100 min

[1]With dissection to highlight ureters.
ND: No data available.

quenching. However, when diluted to 20 mmol/L (quenching threshold) or below, methylene blue displays moderate NIRF[71], with absorption occurring at 668 nm and excitation emission at 688 nm[72]. When the emission wavelength is shorter than that of ICG, the fluorescence of methylene blue is visible less deeply. Because a major route of excretion of methylene blue is through the kidneys, the urinary excretion rate amounts to 28.6% (± 3.0/24 h)[73], and urine becomes NIR fluorescent after a single intravenous dose, which permits real-time identification of the ureters. As the urine flow in the ureter is not continuous but pulsatile and the fluorescence signal is related to urine flow, the fluorescence signal in ureters varies over time. However, a preclinical study using this technique showed that a diuretic, which increased the flux of urine through the ureters, did not increase NIRF[71].

Barnes *et al*[72] showed that 93% of the ureters were seen with NIR techniques. The ureteric fluorescence was bright enough to assure the surgeon that the ureter was positively identified; therefore, no additional dissection was required to identify the ureter under white light. Moreover, 20% of all ureters were visible only under fluorescence and not seen with white light. In laparoscopy, fluorescence was deemed useful; in 10 cases, fluorescence revealed the ureter to be in a place different from the one that the surgeon predicted, and in 2 cases, the surgical plan was subsequently modified. Fluorescence was not deemed useful in open surgery, as tactile feedback is sufficient to detect ureters. The ureters can be seen directly after the injection of methylene blue for more than two hours. According to various studies, the fluorescence time and the visualization quality seemed to depend on the dose and concentration of dye administered.

There were no adverse events observed after the administration of the dye in these studies. However, methylene blue is known to cause severe adverse reactions, such as anaphylaxis, methemoglobinemia or severe haemolysis, in subjects with known glucose-6-phosphate dehydrogenase deficiency[71]. It is important to note that intravenous injection of methylene blue causes an artificial drop in oxygen saturation measured *via* pulse oximetry that lasts up to several minutes. This phenomenon is caused by the principle of pulse oximetry, which is based on the red and infrared light absorption characteristics of oxygenated and deoxygenated haemoglobin.

Recently, experimental dyes have been studied for intraoperative visualisation of ureters using NIRF imaging in laparoscopic surgery. First, IRDye 800CW (LI-COR Biosciences, Lincoln, Nebraska, United States) is a tetrasulfonated heptamethine indocyanine dye that, after intravenous injection, is cleared by the kidneys and excreted into urine. Its maximum absorption occurs at 775 nm, and its maximum

excitation emission occurs at 796 nm, which enables a visualization depth equivalent to ICG. Animal studies[67,74,75] have successfully demonstrated the potential of this dye for the identification of ureters. However, a major disadvantage of IRDye 800CW is its cost, which is almost tenfold that of ICG. In 2018, a new preclinical dye, IRDye 800BK (LI-COR Biosciences), with a maximum absorption of 774 nm and a maximum emission of 790 nm, was developed for NIRF visualization, and its price should be similar to that of ICG. Because of its hydrophilic nature, IRDye 800BK is primarily cleared by the kidneys and enables visualization of the ureters in pigs[76,77].

A comparison of IRDye 800BK *vs* 800CW by Van den Bos *et al*[76] showed that IRDye 800BK successfully identified the course of the ureter in living pig models but also resulted in the highest contrast between the ureter and background. This new dye would allow ureter visualization from 20 min to ≥ 120 min post intravenous injection [77], and the duration and quality of the visualization would be influenced by the concentration of dye. The first study in humans is currently underway (NCT03387410 Thomas Barnes, Oxford University Hospitals NHS Trust).

DISCUSSION

This review of the literature presents the role of NIRF imaging in colorectal surgery. The literature in the field suggests that NIRF imaging is safe and feasible. Furthermore, NIRF imaging seems to be useful for helping the surgeon make decisions during surgical procedures.

NIRF imaging is suitable for all types of surgery but is of particular interest in laparoscopy, where surgeons do not have tactile feedback. Indeed, this modality seems helpful for the detection of ureters to reduce the risk of iatrogenic ureteral lesions. Various dyes have been tested, and a new fluorochrome is under investigation to improve NIR brightness compared to that of methylene blue.

Even if several meta-analyses have been performed for the estimation of intestinal vascularization, very few randomized trials have validated the technique in its different indications. Despite the increase in the number of clinical studies, the interpretation of fluorescence is based on the subjective evaluation of the surgeon, and only a few published studies[19,78] have attempted to determine an objective threshold of fluorescence quantification to normalize the technique on a larger scale. Although some notions of the costs of the methods can be found in the articles read, these data come from heterogeneous studies with small sample sizes and are therefore not comparable. Thus, cost-effectiveness studies would be interesting in the future.

In oncology, the NIRF imaging system is currently used in operating rooms using non-specific fluorescent probes, such as ICG, to visualize SLNs. In recent years, many studies have focused on developing targeted fluorescent probes. Targeted fluorochromes are composed of a carrier molecule that specifically binds to a certain target (*i.e.*, monoclonal antibodies, peptides, small molecules), conjugated to a fluorescent dye. Studies should focus on tumour markers that could provide surgeons real-time feedback about the location and extent of tumours. Seven potential targets for imaging CRC have been identified: CXCR4, EpCAM, EGFR, CEA, Muc1, MMP, and VEGF-A [79], and some have been investigated in other types of cancers. To date, in CRC, two phase I feasibility studies have been performed in humans[80,81], and more molecules have been tested in mice with human CRC xenografts[82-84].

Cancer detection is not the only indication of these new targeted dyes. Neumann *et al*[85] described using an enzymatic marker made by a dye becoming fluorescent only in response to MMP for early identification of AL by targeted fluorescence endoscopy during the healing process. Hingorani *et al*[86] demonstrated in ex vivo human nerves and in vivo mouse models that the fluorescent FAM-HNP401 peptide, when injected intravenously, localized to the connective tissue surrounding peripheral nerves and highlighted these nerves.

CONCLUSION

NIRF imaging appears to be a useful tool to assist surgeons in colorectal surgery. This technique has been tested for three main indications: (1) In the estimation of intestinal vascularization to detect areas of poor perfusion and therefore prevent AL, or in the assessment of the extent of ischaemic segments during acute intestinal ischaemia; (2) In the visualization of lymphatic drainage and (especially in oncological contexts) SLNs and lymphatic and peritoneal metastases in order to prevent recurrence and

adapt further adjuvant treatment; and (3) In the detection of ureters in order to reduce the risk of iatrogenic ureteral lesions, particularly during laparoscopic surgery. Randomized controlled trials and prospective studies with larger sample sizes are needed to validate the methods. Additional studies are underway for the first use of new fluorescent molecules in humans.

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MINIREVIEWS

Dysphagia, reflux and related sequelae due to altered physiology in scleroderma

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Abstract

Systemic sclerosis is a connective tissue disease that presents with significant gastrointestinal involvement, commonly in the esophagus. Dysphagia is a common clinical manifestation of systemic sclerosis and is strongly related to esophageal dysmotility. However, there are multiple other contributing factors in each step in the physiology of swallowing that may contribute to development of severe dysphagia. The oral phase of swallowing may be disrupted by poor mastication due to microstomia and poor dentition, as well as by xerostomia. In the pharyngeal phase of swallowing, pharyngeal muscle weakness due to concurrent myositis or cricopharyngeal muscle tightening due to acid reflux can cause disturbance. The esophageal phase of swallowing is most commonly disturbed by decreased peristalsis and esophageal dysmotility. However, it can also be affected by obstruction from chronic reflux changes, pill-induced esophagitis, or Candida esophagitis. Other contributing factors to dysphagia include difficulties in food preparation and gastroparesis. Understanding the anatomy and physiology of swallowing and evaluating systemic sclerosis patients presenting with dysphagia for disturbances in each step can allow for development of better treatment plans to improve dysphagia and overall quality of life.

Key Words: Systemic sclerosis; Esophageal motility disorders; Deglutition; Deglutition disorders; Gastroesophageal reflux; Esophagitis

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Core Tip: Systemic sclerosis presents with significant gastrointestinal involvement, with dysphagia being a common clinical symptom. Normal swallowing physiology is broken down into the oral phase, pharyngeal phase, and esophageal phase of swallowing; systemic sclerosis can cause disease processes that affect and disrupt each stage of swallowing. We describe the disruptions to swallowing that occur in each phase and potential therapeutic options to alleviate symptoms.

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INTRODUCTION

Scleroderma is a connective tissue disease that may affect any organ system, creating variegated patient-to-patient manifestations. Scleroderma can occur as localized scleroderma or systemic sclerosis (SSc). Localized Scleroderma involves the skin and subcutaneous tissue, but does not manifest with Raynaud phenomenon, digital ischemia, or visceral organ involvement[1]. SSc can be further divided into the limited form, formerly known as CREST syndrome, or the diffuse form. Limited SSc symptomology includes calcinosis cutis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia[1], while diffuse SSc involves widespread cutaneous involvement and earlier, more progressive systemic involvement[1]. The breakdown of types of scleroderma and features of each are shown in Figure 1.

In SSc, gastrointestinal (GI) involvement, specifically esophageal disease, is very common in both limited and diffuse forms, and may be the first manifestation of disease[2]. A previous study evaluating quality of life has shown significant correlation between GI symptoms and lower quality of life scores[3]. Dysphagia, specifically, is a common presentation of GI involvement in SSc[4]. A recent study using screening questionnaires to evaluate physical, functional, and emotional outcomes due to dysphagia shows mild disability in 74% of patients and moderate to severe disability in the remaining 26%[5]. While many studies report that dysphagia is a result of esophageal dysfunction, oral and pharyngeal involvement can also lead to oropharyngeal causes of dysphagia in SSc[5]. Given the significant impact of dysphagia on quality of life in SSc patients, swallowing dysfunction should be thoroughly worked up so it may be treated appropriately[5].

The aim of this study is to review pathophysiology of SSc as well as the physiology of swallowing and reflux to understand the various disruptions that result in dysphagia in SSc. Understanding this will allow for development of more effective therapeutic plans for SSc patients suffering from dysphagia.

PATHOPHYSIOLOGY OF SSC

The pathophysiology of SSc is complex and has not been fully elucidated. SSc is defined by vascularity changes, autoimmunity, and tissue fibrosis[1]. Vascular insult, which can be due to autoantibody effects, infectious or inflammatory causes, environmental exposure, or any number of catalysts, results in activation of endothelial cells. This leads to activation of platelets, which release various factors, such as thromboxane A2 (TXA2), ADP, and transforming growth factor beta (TGF-beta), that promote platelet aggregation and activation of the coagulation cascade. Activated endothelial cells release endothelin 1 (ET-1), a potent vasoconstrictor that also causes leukocyte adhesion, proliferation of vascular smooth muscle cells, and activation of fibroblasts[1]. Under the influence of ET-1 and TGF-beta, endothelial cells undergo trans-differentiation into mesenchymal cells that have a blunted response to vasodilators such as nitrous oxide[1,6]. This milieu contributes to tissue hypoxia and

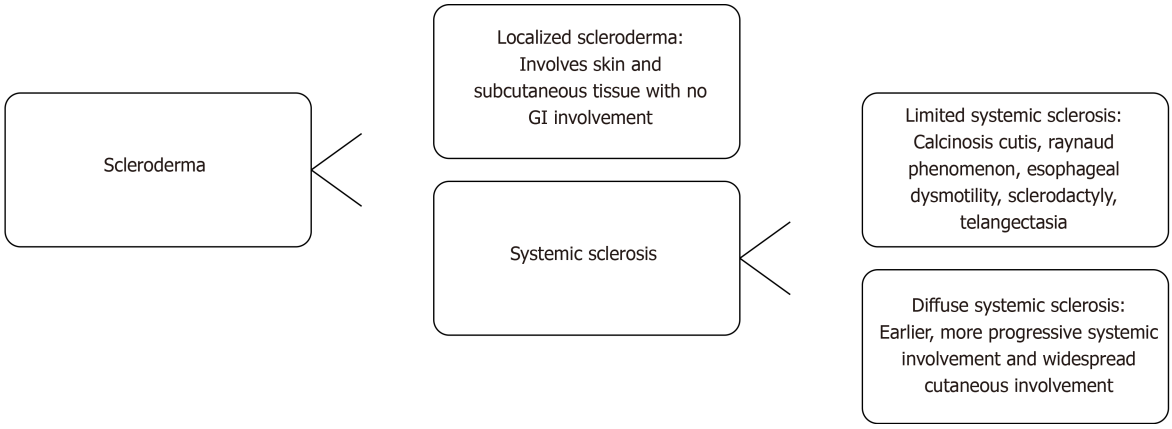


Figure 1 The types of scleroderma and features of each disease. Scleroderma can be broken down into localized scleroderma or systemic sclerosis. Systemic sclerosis can be further broken down into limited and diffuse types. GI: Gastrointestinal.

oxidative stress, exacerbated by diminished angiogenesis secondary to the impaired functioning of endothelial progenitor cells in SSc[1]. Furthermore, SSc may be associated with vascular endothelial growth factor isoforms that are antiangiogenic[6].

Immune system involvement also plays a role in SSc pathogenesis, with dysregulation of both innate and humoral immunity. SSc presents an imbalance of T helper cells defined by a predominance of type 2 T helper (Th2) cells[1]. The cytokines produced by these overexpressed Th2 cells, including IL-4 and IL-13, are pro-fibrotic [7]. Th2 cells also induce immunoglobulin production by B cells; these upregulated autoantibodies contribute to inflammation. Limited SSc is associated with anti-centromere antibodies while the diffuse form is associated with anti-Scl-70 antibodies or anti-DNA topoisomerase I antibodies, as well as anti-RNA polymerase III antibodies. There is an association between anti-RNPC3 antibodies and severity of SSc GI disease, with the presence of these antibodies predictive of moderate or severe GI disease. The subunit of RNA polymerase III that is targeted by autoantibodies can further stratify GI severity in SSc patients[8]. Activation of the immune system in this way leads to fibrosis. The excess collagen produced by myofibroblasts contributes to this process of structural damage[7]. The basic pathophysiology of SSc is outlined in Figure 2.

Autonomic dysfunction may also contribute to SSc symptomology and severity. Vascular damage to the vasa nervorum is important in the development of this dysfunction[9]. Autoantibodies against nicotinic acetylcholine receptor at autonomic ganglia (gAChR) may also play a role in GI autonomic dysfunction, as anti-gAChRα3 autoantibodies were significantly higher in patients with SSc GI disease than in SSc patients without GI manifestations[10]. Anti-muscarinic-3 receptor autoantibodies may also contribute by inhibiting contraction of smooth muscle cells[11].

SSc esophageal involvement

GI involvement, specifically esophageal involvement, is the most common internal organ complication of SSc, affecting over 90% of patients[8]. While patients with the diffuse form have greater skin and muscular involvement, there is no difference in GI involvement between the diffuse and limited forms of SSc[12]. Since smooth muscle is targeted in the pathophysiology of SSc, the lower two-thirds of the esophagus and lower esophageal sphincter (LES) are preferentially affected, as the upper third of the esophagus is primarily composed of skeletal muscle. Esophageal symptomatology typically includes dysphagia, pseudo-obstruction, regurgitation, heartburn, nausea, or vomiting, all of which can lead to poor eating and severe weight loss[7]. Esophageal dysfunction permits gastric reflux, which can lead to erosive esophagitis, bleeding, and ulceration, as well as eventual stricture and fistulae formation and an achalasia-like syndrome. Barrett's esophagus and adenocarcinoma may result from long-term disease. Micro-aspiration due to esophageal dysmotility is also associated with interstitial lung disease (ILD)[7].

Diagnostic delay may occur in SSc GI disease since 30% of patients with esophageal involvement may be asymptomatic, especially early in the disease course[7]. Various diagnostic studies can be used in detection of esophageal disease including manometry, pH monitoring with or without impedance, and esophagogastroduodenoscopy (EGD)[4]. Radiographic imaging may also be used: Videofluorography

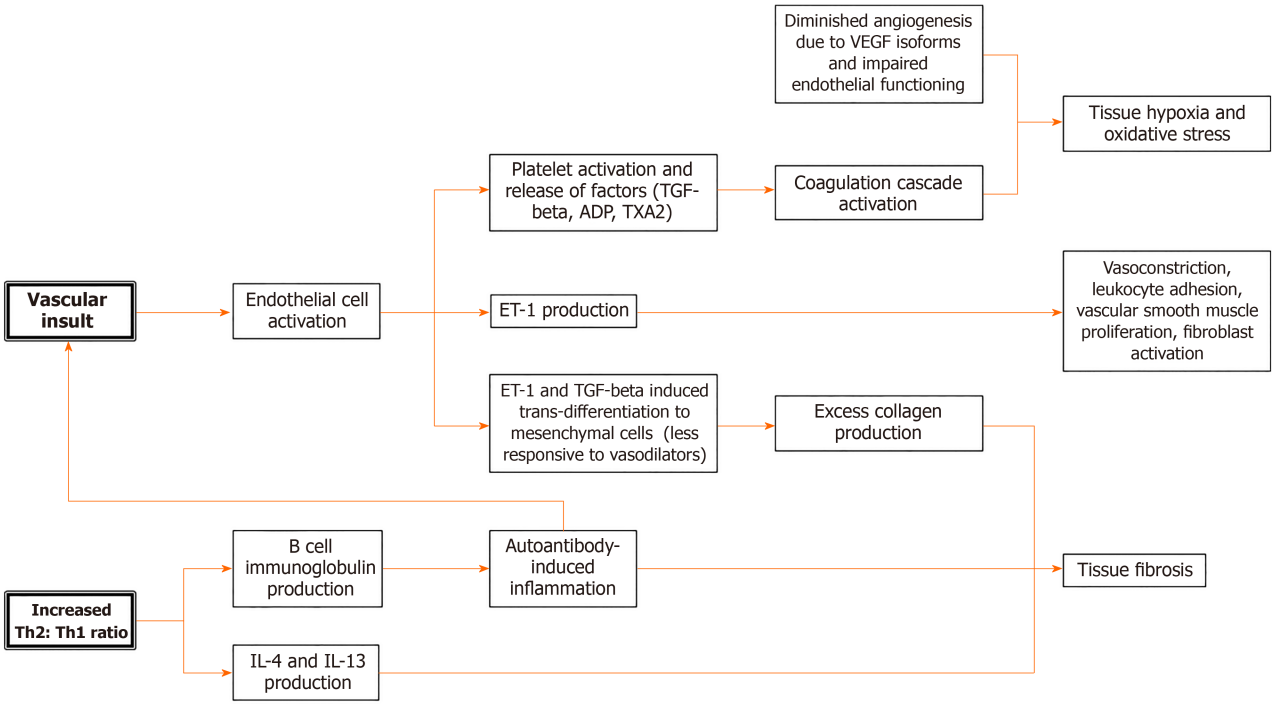


Figure 2 Simplified pathophysiology of systemic sclerosis. SSc: Systemic sclerosis; Th2: Type 2 T helper; Th1: Type 1 T helper; TGF-beta: Transforming growth factor beta; TXA2: Thromboxane A2; ET-1: Endothelin 1; VEGF: Vascular endothelial growth factor; IL: Interleukin.

swallow study of the esophagus can also show altered peristalsis and a standard chest computed tomography (CT) may reveal a dilated esophagus[13]. The diagnostic studies are outlined in Table 1. When patients are symptomatic, associated GI symptoms can be scored in various ways according to symptomology. UCLA Scleroderma Clinical Trial Consortium GIT 2.0 (UCLA SCTC GIT 2.0) is a scoring system used to assess GI tract symptom severity as well as health-related quality of life [14]. The scoring system has 34 questions assessing 7 different scales, which include reflux, distention/bloating, diarrhea, fecal soilage, constipation, emotional well-being, and social functioning, as outlined in Table 2[14]. The score is based on a questionnaire that asks about severity and frequency of symptoms, ranging from "no gut symptoms" to "very severe symptoms"[14]. This scoring system is found to be reliable and feasibly applicable as it takes only 6-8 min to complete[14]. The UCLA STCT GIT 2.0 is very useful in SSc patients not only for evaluating severity of GI disease but also for following improvement in symptoms with various treatments[14].

SSc patients, especially those with diffuse SSc in which complications typically occur more rapidly, report adverse effects of the disease on their quality of life. Patients describe fatigue, manual activity limitations, distress related to manifestations and unpredictability of their disease, sleep difficulties, and low self-esteem; patients do not feel fully equipped to handle their disease mentally or physically[15]. Anxiety related to the disease is prominent in the SSc population, further dampening quality of life[15]. The prevalence of GI disease in SSc, as well as the harsh symptomology that can result from GI forms of the disease, likely makes GI involvement a contributor to poor quality of life in these patients.

PHYSIOLOGY OF SWALLOWING

Dysphagia is common in SSc and may be related to disease affecting the smooth muscle of the distal two-thirds of the esophagus and the LES as well as other disease processes in SSc[1,16]. It is therefore necessary to first review the normal swallowing physiology to understand how various manifestations of SSc can cause disruption to each step of this process. Physicians can then apply this knowledge clinically by recommending treatment plans targeting these factors through lifestyle changes, medications, and procedures to improve dysphagia symptoms and quality of life.

Table 1 Features of various diagnostic studies in systemic sclerosis diagnosis

Diagnostic study	Role in SSc
Esophagogastroduodenoscopy	Evaluates for esophageal causes of dysphagia[4]
	Shows reflux-related complications: erosive esophagitis, strictures, Barrett's esophagus, esophageal adenocarcinoma[4]
	Reveals esophageal findings in asymptomatic patients[4]
Esophageal manometry	Detects esophageal dysmotility, even in early stages of SSc[4]
	Shows decreased lower esophageal sphincter pressure and absent peristalsis in distal two-thirds of esophagus[4]
Pharyngeal manometry	Evaluates for oropharyngeal dysphagia by assessing upper esophageal sphincter relaxation and pharyngeal propulsion[38]
Esophageal pH monitoring (with or without impedance)	Gold standard for gastroesophageal reflux detection[4]
	Used for patients with resistant reflux[4]
Videofluorography swallow study of esophagus	Shows esophageal dysmotility with decreased peristalsis in distal 2/3 of esophagus[13]
	Shows decrease of lower esophageal sphincter pressure[13]
	Shows dilated lumen of esophagus[13]
CT chest	Shows esophageal dilation[13]

SSc: Systemic sclerosis; CT: Computed tomography.

Table 2 Scoring system of gastrointestinal symptoms based on UCLA Scleroderma Clinical Trial Consortium GIT 2.0[14]

Scales	None-to-mild symptoms	Moderate symptoms	Severe-to-very severe symptoms
Reflux			
Distension/bloating			
Diarrhea			
Constipation			
Fecal soilage			
Emotional well being			
Social functioning			
Total GIT score			

Swallowing is a coordinated, physiological process that is important for consumption of food and fluid. It is essential for sustenance and any disruption in it affects perception of quality of life[17]. Difficult or disordered swallowing can cause further medical complications such as malnutrition, dehydration, and aspiration pneumonia[17,18].

The physiological process of swallowing is broken down into three stages: the oral stage, the pharyngeal stage, and the esophageal stage[17]. The oral stage is the voluntary first step, and involves the lips, teeth, muscles of mastication, and tongue [17]. In the oral preparatory phase of the oral stage, mastication allows the breakdown of food as it is moved around the oral cavity and lubricated with saliva[17,19]. During the propulsive stage of the oral phase, the food bolus is first positioned on the superior surface of the tongue[17]. Tongue flexion beginning anteriorly and moving posteriorly pushes the bolus towards the pharynx, to begin the second stage of swallowing[17]. In the pharyngeal phase of swallowing, the larynx and soft palate move upwards to block off the airway and nasopharynx[19]. Several muscles of the anterior portion of the pharynx contract to cause forward displacement of the larynx and pharynx[19]. This is followed by relaxation of the cricopharyngeal muscle and relaxation and opening of the upper esophageal sphincter (UES)[19]. As the UES opens, the bolus will pass through to the esophagus, beginning the esophageal phase of swallowing[20]. Once the bolus enters the esophageal lumen, it is transported by coordinated,

sequential muscular contractions and relaxations known as peristalsis[19]. This peristalsis is triggered by distension of esophagus and allows for the bolus to travel towards the stomach[19]. This primary peristalsis may be followed by a secondary wave. Secondary peristalsis is limited to the smooth muscle portion in the bottom 2/3 of the esophagus, and functions to clear remnants of the bolus leftover from the primary wave and to remove refluxed gastric contents[19]. The final step in the esophageal phase of swallowing is relaxation of the LES and movement of the bolus into the stomach[19]. Relaxation occurs in a coordinated manner with the preceding peristalsis[19]. The LES remains tonically contracted at rest and is supported by the crural diaphragm[19].

Dysphagia, defined as difficulty in swallowing that results in delay in passage of food or liquid bolus[21], is a common symptom in SSc patients due to various disturbances in the normal swallowing process[1]. In these patients, each stage of swallowing can be disrupted in various ways, each leading to a different classification of dysphagia[21]. Good history-taking followed by necessary diagnostic testing in SSc patients can define the type of dysphagia so an appropriate therapeutic plan may be recommended. The oral phase of swallowing in SSc may be disrupted by loss of teeth and microstomia limiting mastication. Impaired saliva production may further complicate the oral phase. Pharyngeal dysphagia commonly occurs due to uncoordinated contractions, muscular weakness causing decreased contraction, or anatomic anomalies causing obstruction[21]. In SSc, there may be weakening of pharyngeal muscles due to concurrent myositis or cricopharyngeal muscle tightening secondary to reflux. Esophageal dysphagia is commonly due to mechanical obstructions or motility disorders[21]. Dysmotility characterized by decreased peristalsis or obstruction secondary to chronic reflux changes may result in esophageal dysphagia in SSc. The various disease processes affecting each stage of swallowing in SSc are outlined in Figure 3. The potential treatment options for these processes are outlined in Table 3.

CAUSES OF ORAL DYSPHAGIA IN SSC PATIENTS

Pre-oral phase food preparation

Hand function impairment in SSc can commonly lead to difficulty in food preparation and presentation. Hand dysfunction in SSc is widely recognized and is typically due to skin thickening and contracture of the hands. Tests such as the Hand Mobility in Scleroderma (HAMIS) test are frequently used to assess finger and wrist mobility[22]. In a study of 30 patients with SSc, it was found that hand dexterity was reduced to 68%-80%, and grip force was reduced to 46%-65% compared to a healthy person. Finger flexion and extension were the most impaired in hand mobility[23]. Ischemic digital ulceration in SSc also contributes to impairment of hand function as well as increased pain[24]. These impairments lead to difficulty in preparing or ingesting food in patients with SSc. These quality-of-life changes affecting eating are addressed in the Scleroderma Health Assessment Questionnaire, which asks questions such as "Are you able to cut your meat?" and "Are you able to lift a full glass to your mouth?"[25]. Those who do endorse difficulty cutting their food may have further swallowing symptoms due to ingestion of larger pieces of food. Inability to lift a glass can also limit water intake, that would have facilitated swallowing.

Disruption in mastication and food bolus preparation

The oral voluntary phase of swallowing may be also severely limited in SSc due to various factors that can subsequently impact the following swallowing phases. This adds to the burden of diminished esophageal motility function and dysphagia in SSc patients. Studies show that SSc patients may suffer from microstomia due to sclerofibrosis of perioral tissue and malfunctioning of the temporomandibular joint [26]. This limits SSc patients' ability to open their mouths beyond 40 mm, and results in limited mandibular movements[27]. This causes disturbances in mastication and food bolus preparation, affecting the oral preparatory stage of the oral phase of swallowing and contributing to dysphagia in SSc patients.

Other disturbances in SSc lead to poor dental hygiene in this patient population. Teeth enamel can be eroded due to gastric acid reflux[2] and xerostomia[28]. In addition, SSc patients may have sclerodactyly and hand dysfunction, which can limit their ability to brush and care for their teeth[26]. This results in dental cavities, decay, and ultimately loss of teeth, and causes disruption in chewing of the food bolus for mastication in the oral phase of swallowing[26]. Other findings in SSc patients include

Table 3 Treatment options for the various disease processes that contribute to the development of dysphagia in systemic sclerosis

Disease process	Therapeutic plan
Xerostomia	Drinking water more frequently[2]
	Using artificial saliva as needed[2]
	Using special toothpastes and mouthwashes[2]
	Avoiding medications that exacerbate dry mouth[2]
Microstomia	Performing exercises and massages to stretch the mouth[2]
Dental decay	Planning regular follow-up with experienced dentist[2]
Concurrent myositis	Frequent screening for myositis in patients with SSc and suggestive symptoms[37]
	Treating concurrent myositis with immunomodulatory therapy and interventional procedures[37]
Esophageal Dysmotility	Lifestyle management (taking smaller bites, chewing food thoroughly, drinking adequate water with food)[16]
GERD	Medications (PPIs, H2RAs)[2,52]
	Dietary modifications (avoiding acidic foods)[52]
	Lifestyle modifications (avoiding meals before bedtime, elevating the head of the bed while sleeping)[2,57]
Candida esophagitis	Screening for fungal esophagitis in patients with SSc and suggestive symptoms[73]
	Prompt antifungal treatment[73]
Pill esophagitis	Avoiding medications at high risk of causing esophagitis[79]
	Screening for esophagitis in SSc patients taking culprit medications with suggestive symptoms[79]
Gastroparesis	Medications (prokinetic agents)[2]
	Dietary modifications of small frequent meals with fiber[2]

SSc: Systemic sclerosis; PPIs: Proton pump inhibitors; H2RAs: H2-receptor antagonists; GERD: Gastroesophageal reflux disease.

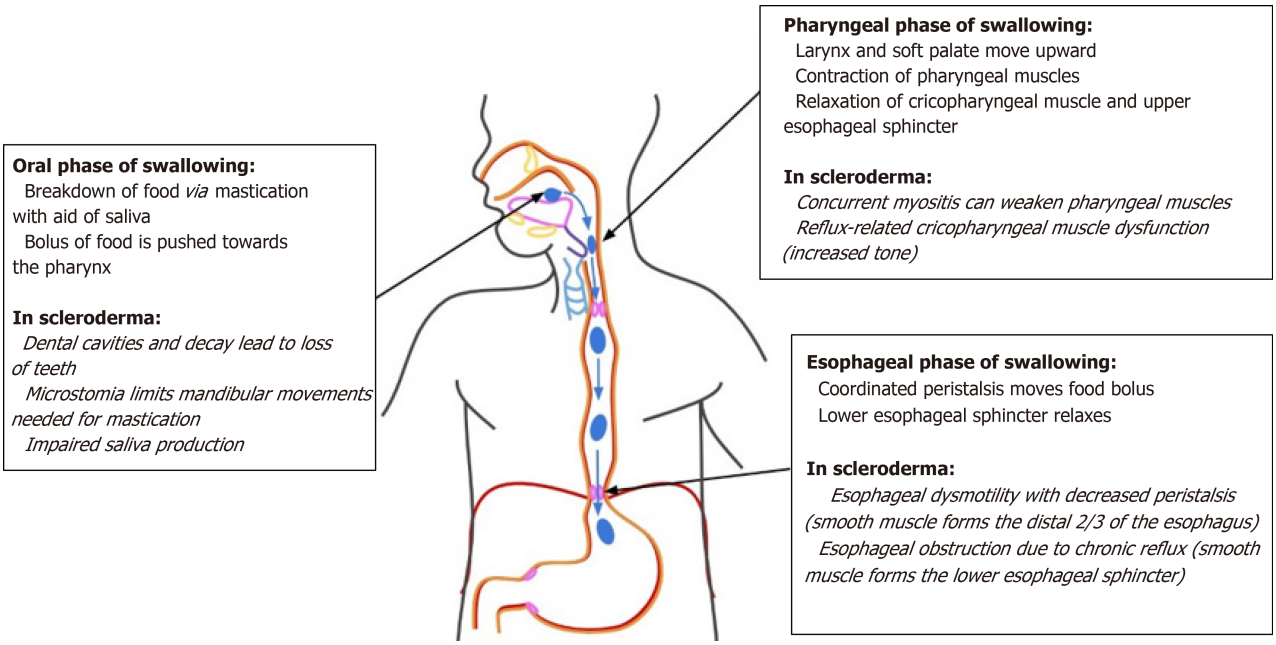


Figure 3 Disruptions of normal swallowing physiology due to disease processes of scleroderma.

widening of the periodontal ligament around the premolar and molar teeth[29], mandibular bone resorption[26], and gingival recession[26].

There are very few treatments to ameliorate the issue of poor dentition in SSc. Typical treatments for poor dentition include dentures and implants, which are not feasible in SSc. Dentures are difficult to apply due to impaired hand function as well as

oral cavity narrowing and tongue rigidity in SSc patients[30]. Once applied, salivary hypofunction may also reduce denture retention[30]. Implants are also difficult due to low bone mineral density in SSc patients[31]. Mandibular bone resorption, commonly seen in SSc patients, is thought to be secondary to pressure ischemia from sclerosed skin and muscle atrophy[32]. Proper dental care and hygiene is essential in preventing and treating dysphagia in SSc patients. Patients may require assistance in brushing their teeth and should be encouraged through routine checks with experienced dentists.

Impaired saliva production and concomitant Sjogren's syndrome

Patients with SSc have a higher prevalence of concomitant Sjogren's Syndrome, an autoimmune disease characterized by inflammation of exocrine glands resulting in dryness of mucosal surfaces. Specifically, there is a loss of salivary glands that leads to xerostomia[33]. Sjogren's disease is seen in 17%-29% patients with SSc[33]. Saliva is necessary for mastication, digestion, oral cleansing, and speech articulation[34]. It contains water, minerals, electrolytes, buffers, and proteins that maintain neutral pH and aid in enzymatic digestion. Loss of the bicarbonate buffer contained within saliva disrupts one defense mechanism against acid reflux, and may result in more severe gastroesophageal reflux disease (GERD) symptoms[19]. Salivary dysfunction also impairs the oral preparatory stage of the oral phase of swallowing, as saliva is needed for lubrication of food and mastication[17,35]. This disturbance may contribute to the overall prevalence of dysphagia in SSc patients.

CAUSES OF PHARYNGEAL DYSPHAGIA IN SSC PATIENTS

Concomitant myositis

Patients with SSc are more prone to suffering from concurrent myopathies that may contribute to the pathophysiology of dysphagia. Prior studies note anywhere between 5%-81% of SSc patients suffer from myopathies, depending on the definition of muscle involvement. Myositis in patients with SSc is termed as Scleroderma Polymyositis Overlap. Scleroderma Polymyositis Overlap is characterized by a more active fibrotic disease and is associated with an increased rate of systemic complications as well as a higher mortality rate[36]. Polymyositis is a myopathy characterized by muscle impairment and systemic symptoms secondary to muscle inflammation of striated skeletal muscles[37]. Because coordinated contraction of the striated skeletal muscles of the oropharynx and upper esophagus is essential to the swallowing process, inflammation and dysfunction of these muscles, as seen in polymyositis, can cause pharyngeal dysphagia[37]. Dysphagia is reported in up to 36% of patients with myositis[37]. On pharyngeal high-resolution impedance manometry, there may be globally weak pressure generation, with absent hypo-pharyngeal constrictor activity as well as abnormal UES contractility[38]. These findings correlate with increased post-swallow residue and aspiration risk[38]. Given the risk of concomitant myositis in SSc patients, pharyngeal dysphagia due to striated muscle weakness should be considered a potential cause of dysphagia in SSc patients. The possibility of additional disruption in the pharyngeal phase of swallowing may result in a more severe dysphagia in SSc patients.

Cricopharyngeal muscle disorder

Cricopharyngeal muscle disorders can commonly develop in SSc patients due to changes from chronic reflux. A previous study found that 51% of patients with gastroesophageal reflux have pharyngoesophageal dysphagia due to cricopharyngeal muscle dysfunction[39]. Another study in which HCl was infused proximal to the gastro-esophageal sphincter revealed an increase in UES tone 1 min after infusion, revealing the reactivity of the cricopharyngeal muscle to acid reflux[40]. Cricopharyngeal muscle dysfunction resulting in increased tone may prevent passage of food bolus from pharynx through UES into the esophagus. Given the high prevalence of reflux in SSc patients, cricopharyngeal muscle dysfunction may be an important complication to consider. Several case reports have also shown SSc patients specifically presenting with UES stenosis[41,42]. This upper esophageal involvement in SSc is an important contributor to dysphagia during the pharyngeal phase of swallowing.

CAUSES OF ESOPHAGEAL DYSPHAGIA IN SSC PATIENTS

Esophageal dysmotility

SSc commonly presents with GI involvement, manifested primarily in the esophagus as dysmotility[16]. The distal two-thirds of the esophagus is affected due to its smooth muscle makeup[2]; this area of smooth muscle is likely commonly cited as the first affected location of the GI tract because esophageal changes are readily sensed by patients due to the daily frequency of swallowing. While loss of peristalsis in other areas of the GI tract may become noticeable to patients with time, the fact that swallowing is under partial voluntary control and performed multiple times per day presumably highlights any associated discomfort. Further, perhaps the two-layered muscularis of the esophagus is more quickly affected than the thicker three-layered muscularis of the stomach, for example[43].

Prospective studies show esophageal dysmotility confirmed *via* manometry in about 70%-75% of SSc patients[16]. Esophageal involvement results in decreased amplitude of esophageal peristalsis as well as decreased tone of the LES. Esophageal manometry can confirm scleroderma as the cause of esophageal dysmotility by showing absent peristalsis in the distal two-thirds of the esophagus and a hypotensive LES[44], as seen in Figure 4. Clinically, patients most commonly present with dysphagia to both solids and liquids[16]. The lack of peristalsis in scleroderma results in esophageal dysphagia due to a motility issue, as lack of coordinated contractions/relaxations in the esophagus prevent food from being efficiently transported to the stomach[21]. Other clinical symptoms related to esophageal dysmotility in scleroderma include globus sensation, reflux, heartburn, or chest discomfort with swallowing[16]. Complications related to esophageal dysmotility include gastroesophageal reflux, stasis of foods in the esophagus, and aspiration into the lungs[16]. While there are no medications that effectively treat esophageal dysmotility, symptoms can be alleviated by lifestyle management. This includes taking smaller bites, chewing food thoroughly, and hydrating with water when having any solid, dry foods[16]. While esophageal dysmotility is commonly thought to contribute to dysphagia in SSC[1], the development and progression of further complications can cause more severe disruption to the normal swallowing physiology and may worsen dysphagia symptoms.

Gastroesophageal reflux

In individuals with a normal esophagus, throughout the day, only small amounts of gastric contents reflux into the esophagus due to presence of multiple defensive barriers[19]. These include inherent LES tone with surrounding muscles, like the crural diaphragm, augmenting tone, secondary peristalsis to rapidly clear refluxed materials, bicarbonate in swallowed saliva to neutralize acid, local bicarbonate and mucus secretion, and resistance of the epithelium to degradation[19]. In patients with GERD, gastric contents with acid or pepsin reflux into the esophagus and cause inflammation or esophageal injury, possibly due to defective defensive mechanisms[19]. If the LES is impaired by disease or inflammation, it may allow contents to reflux. Defective peristalsis may limit clearance of contents, causing further injury and inflammation with longer contact times[19]. In addition, reduced salivation will decrease neutralization of refluxed contents[19]. Certain anatomic irregularities can also worsen reflux frequency and severity. For example, hiatal hernias decrease the overlap between the crural diaphragm and LES, thereby decreasing LES tone as it is no longer supported by the diaphragm[19,45]. In addition, transient LES relaxation (TLESR) has a major role in GERD pathology[19,45]. Physiologically, TLESRs occur after meals as they are triggered by gastric distension to allow the release of gas from the stomach as belching [45]. In patients with GERD, TLESR can allow acid in addition to gas to be refluxed into the esophagus[45]. Studies have shown that most episodes of acid reflux occur during TLESRs[46]. In patients with GERD, TLESRs occur more frequently, acid refluxes into the esophagus more often, and refluxed contents reach more proximal levels in the esophagus than in patients without GERD[46].

GERD is a very common diagnosis in SSc patients due to various disruptions of reflux defense mechanisms, as outlined in Figure 5. Based on large retrospective studies, the prevalence of esophageal reflux in SSc patients is 34.8%[2,47]. Clinically, patients report classic heartburn and reflux symptoms as well as dysphagia, odynophagia, laryngitis, chronic cough, hoarseness, or asthma[16]. In SSc, esophageal dysmotility prevents secondary peristalsis from efficiently clearing acidic refluxed materials[16], highly predisposing SSc patients to developing severe GERD[2]. The loss of tone in the LES also contributes to the development of GERD and complications from reflux[2]. GERD-related complications such as reflux esophagitis, strictures,

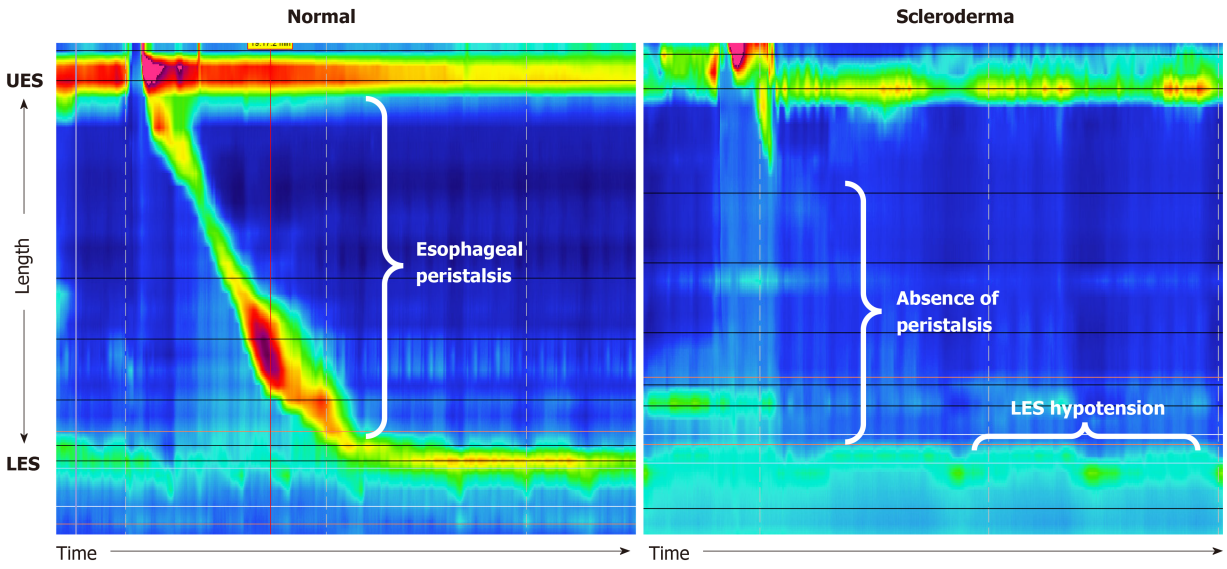


Figure 4 Normal manometry findings showing peristalsis during swallowing and lower esophageal sphincter tone compared to manometry findings in scleroderma showing an absence of peristalsis and lower esophageal sphincter hypotension. LES: Lower esophageal sphincter; UES: Upper esophageal sphincter.

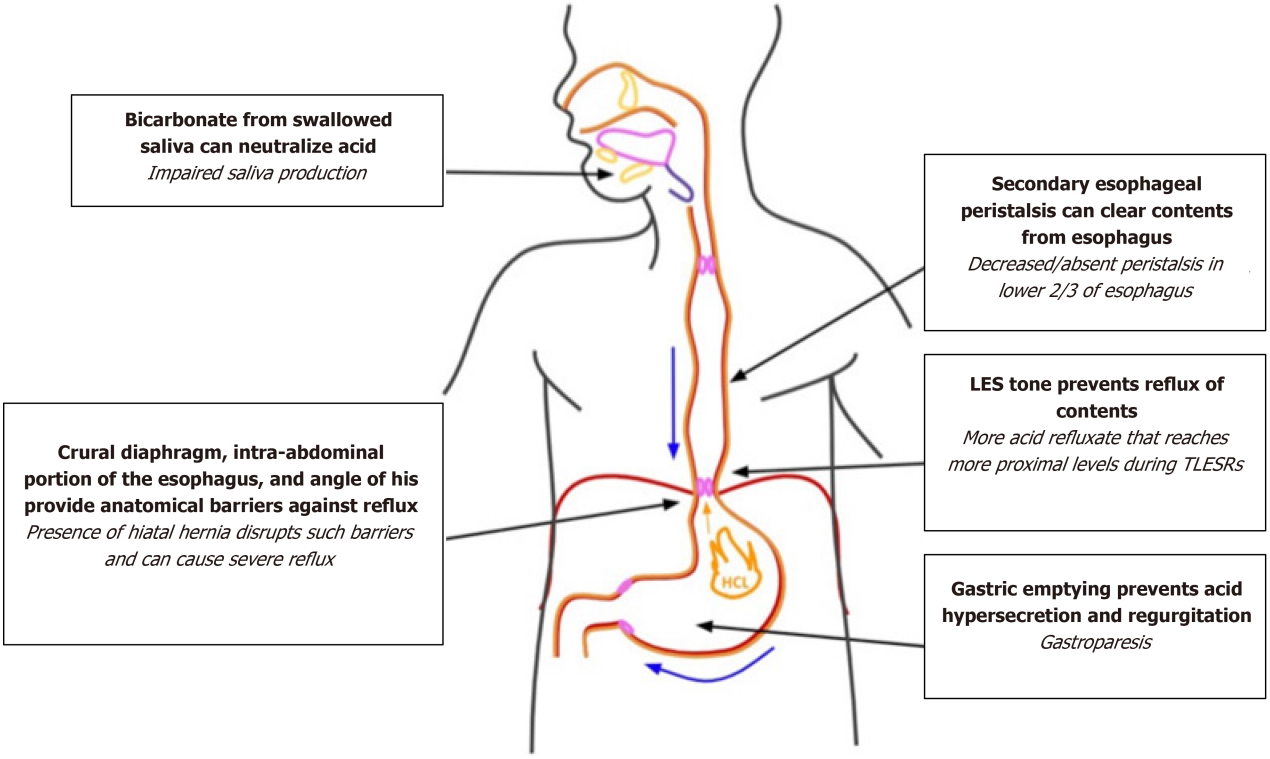


Figure 5 Disturbances of defense mechanisms against reflux in scleroderma. LES: Lower esophageal sphincter.

Barrett's esophagus, and adenocarcinoma may cause further dysphagia symptoms[2]. Gastroparesis (Gp) and small bowel dysmotility are also common symptoms in scleroderma and may worsen GERD symptoms[48]. SSc is also associated with impaired saliva production, which impedes the ability to neutralize the refluxed contents[48].

The presence of hiatal hernia in SSc patients may exacerbate GERD symptoms as well. One study comparing 25 SSc patients to 25 control patients found presence of hiatal hernia in 16 out of 25 SSc patients and only 3 out of 25 control patients[49]. Another cross-sectional study of SSc patients found that those with gastroesophageal symptoms had a significantly higher frequency of hiatal hernia than asymptomatic

patients[50]. Concurrent hiatal hernia likely contributes to the occurrence and severity of GERD symptoms in SSc patients. Loss of multiple defense mechanisms against reflux through the various disease processes of SSc may explain why SSc-related GERD is associated with more severe symptoms and more complications[51].

In SSc patients that present with GERD, treatment is imperative to prevent development of acid-induced complications. Medical management *via* proton pump inhibitors (PPIs) is effective in relieving GI symptoms[16]. H2-receptor antagonists (H2RAs) can also be used, though less effective than PPIs[16,52]. This medication therapy should be coupled with lifestyle measures. This includes avoiding acidic foods, abstaining from drinking alcohol and smoking, losing weight, eating smaller meals, and elevating the head of the bed[52].

TLESR

TLESRs are important in development of GERD[45], as studies indicate that TLESRs may be more important than intervals of persistently low LES tone for reflux episodes [45,46]. Given that TLESRs are triggered by gastric distension[45], they may occur more frequently in SSc patients who suffer from Gp-related distension. In patients with GERD, reflux during TLESRs was found to be more acidic and reach more proximal levels of the esophagus than in normal patients[46]. This was thought to be related to decreased esophageal contraction in response to acid reflux[46]. Loss of esophageal motility in scleroderma patients may predispose patients to more acid reflux than can reach more proximal levels of the esophagus during TLESRs. The increase in frequency of TLESRs with refluxate that can reach more proximal levels of the esophagus likely increases severity of GERD symptoms in SSc patients. There has been some evidence suggesting that baclofen may decrease incidence of TLESRs, thereby reducing the number and length of reflux episodes in patients with GERD[16, 53].

Delayed esophageal clearance

Despite the significant overlap between the clinical presentations of SSc esophagus and GERD, there are additional mechanisms in SSc pathophysiology to consider. While GERD is primarily due to reflux of acidic gastric contents up into the esophagus, SSc esophagus has reflux coupled with the stasis of acidic contents due to delayed esophageal clearance, exacerbating symptoms[19,54]. Consequences of delayed esophageal clearance secondary to dysmotility include both severe GERD symptoms from esophageal stasis-related acidity and a dilated distal esophagus[54].

It is important to recognize patients who present with refractory GERD symptoms despite adequate treatments and suspect SSc as a possible diagnosis. In these patients, an EGD will show a dilated esophagus with retained secretions and saliva suggestive of esophageal stasis, and CT may confirm dilated esophagus[54]. Twenty-four-hour ambulatory pH monitor with impedance monitoring and manometry may demonstrate ineffective esophageal motility with subsequent stasis of acidic contents in the esophagus, by documenting prolonged esophageal acidity compared to acid exposure in the stomach[54]. Findings indicative of esophageal stasis on 24-h impedance pH monitoring may be seen in Figure 6. If delayed esophageal clearance is noted through diagnostic testing, it should be addressed promptly, as it is likely a major exacerbator of esophageal acidity. Esophageal stasis of acidic contents may contribute to the severity of GERD symptoms and high rate of complications in SSc patients[51], and should therefore be addressed in therapeutic plans. It is also important to note that esophageal involvement of SSc is linked to development of ILD, a major cause of morbidity and mortality in SSc[2]. ILD severity has been shown to be associated with more active reflux disease, but development may be possible even in SSc patients with esophageal dilation but no clinical esophageal symptoms[2,55]. A retrospective cross-sectional study has shown that the diameter of the esophagus in SSc patients correlates with progression of ILD, as measured by diffusion capacity of the lung for carbon monoxide[2,56]. Given that delayed esophageal clearance can result in both increased acid damage to the esophagus and development of ILD, developing an appropriate treatment plan is essential. Possible lifestyle modifications to consider include eating smaller meals, remaining in an upright position after eating, elevating the head of the bed while sleeping, and avoiding eating in the three hours before sleeping[57,54]. These changes utilize the effect of gravity to promote esophageal clearance of acidic contents[54]. Lifestyle changes can be supplemented with medical therapy targeting esophageal acidity[57].

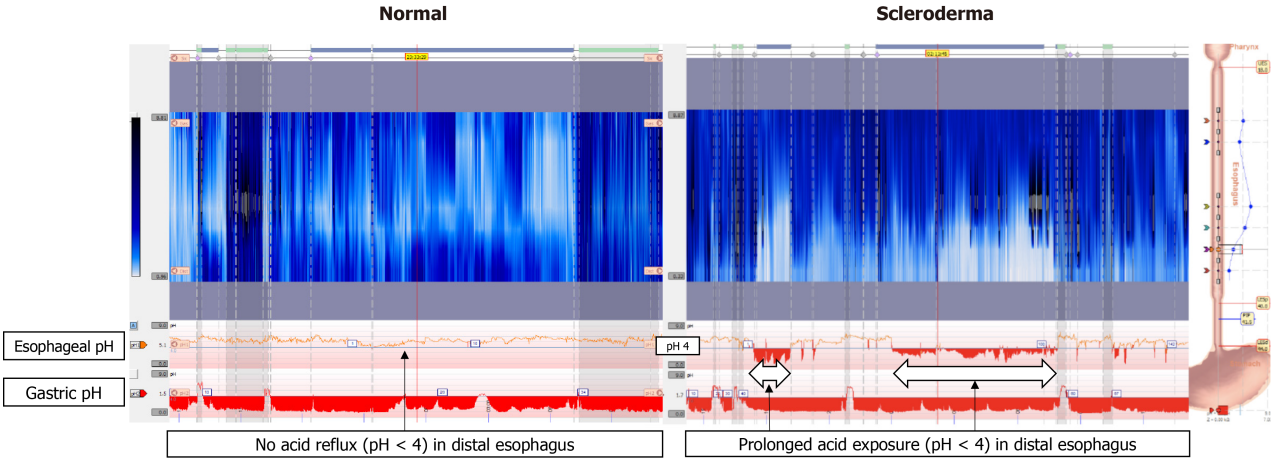


Figure 6 Twenty-four hour impedance pH monitoring normal findings compared to findings in scleroderma. In normal esophagus, there are no episodes of acid reflux, as indicated by no drops of pH below 4 in the esophagus. In scleroderma esophagus, there is prolonged acid exposure in the distal esophagus.

Erosive esophagitis

SSc patients are more prone to developing erosive esophagitis as a complication of severe GERD, which can further contribute to their dysphagia. A collection of studies report that 30%-60% of SSc patients develop erosive esophagitis[58-60]. One study found that 24.2% of SSc patients had mild to moderate erosive esophagitis while 3.6% of patients had severe erosive esophagitis[61]. Mechanisms for development of this pathology all pertain to impaired acid clearance in the esophagus and increased acid exposure, such as impaired peristalsis, impaired LES tone, and pathologic nighttime gastroesophageal reflux[58,59]. Symptoms of dysphagia were also found to correlate with erosive esophagitis[58]. This is likely related to both the development of the erosions in esophagitis as well as their sequela, such as strictures.

Strictures and mechanical obstruction

Strictures are a large contributor to dysphagia in SSc and are found in 17%-29% of SSc patients[62]. A diagnosis of SSc increases the odds ratio of developing strictures to 12 [62]. Strictures result from excess deposition of collagen during healing of erosive esophagitis and are often due to chronic gastroesophageal reflux[57]. The presence of these strictures leads to an obstructive dysphagia as they narrow the esophageal lumen and reduce esophageal distensibility[63]. This leads to further impairment of acid clearance from the esophagus, resulting in more esophageal insult and potential for dysphagia. Schatzki rings have also been found in SSc patients, with one study finding 12.5% of SSc patients with the lower esophageal rings. Though they may be more amenable to treatment *via* dilation, these rings also contribute to dysphagia[64]. However, obstructive dysphagia is not always due to strictures and Schatzki rings in SSc — though rare, adenocarcinoma may also cause obstructive esophageal dysphagia.

Barrett's esophagus and esophageal adenocarcinoma

Reflux-related disorders such as SSc are naturally conducive to the development of Barrett's esophagus. Pathophysiology of Barrett's esophagus is tied to increased acidic insult of esophageal epithelium. Clinically, patients with Barrett's esophagus tend to have longer durations of dysphagia and relatively impaired LES tone[65]. It is unclear whether this association is causation or correlation, as risk factors for Barrett's esophagus may predispose patients to other causes of dysphagia as well[65]. Barrett's esophagus has been found in up to 37% of patients with SSc[62], and is more likely to develop in patients with limited SSc[65]. Patients with SSc that have Barrett's esophagus have an increased risk of developing further complications, such as strictures and adenocarcinoma[66]. Esophageal adenocarcinoma specifically may develop as a result of metaplasia and dysplasia found in Barrett's esophagus. Development is more common in SSc patients, as esophageal adenocarcinoma has an incidence of 0.7% per year in patients with SSc and Barrett's esophagus, while incidence is only 0.45% per year in patients with Barrett's esophagus without SSc[67]. Though other complications of SSc may have higher incidence, esophageal adenocarcinoma should still be considered and ruled out in patients presenting with dysphagia.

Esophageal cancer most commonly presents with mechanical obstruction leading to esophageal dysphagia, so it must be considered as a potential contributor to dysphagia symptoms in SSc patients[66].

Sequelae of SSc treatment

Candida, an opportunistic fungus, is normally a symbiont of the esophagus that rarely gives rise to severe disease indications. Yet *Candida* infection remains the most common cause of infectious esophagitis, with an incidence of up to 88%[68,69]. In the setting of an impaired host-defense system, uncontrolled proliferation of *Candida* in the esophageal mucosa can occur, leading to the formation of characteristic adhesive plaques, which are visualized by endoscopy. Studies have reported that Candida esophagitis (CE) can be a result of poor emptying of the esophagus, acid suppression, and immunosuppressive therapy[62]. Connective tissue diseases, such as SSc, can promote esophageal stasis and can, therefore, lead to fungal colonization of the esophagus. In addition, acid suppression *via* PPIs and H2RAs are commonly used in SSc patients with complaints of GERD[2]. For other various manifestations of SSc such as ILD, immunosuppression therapy is commonly prescribed[70]. As a result, CE and secondary stricture formation is likely to occur with SSc, further complicating dysphagia and esophageal dysfunction in these patients[71]. Patients with CE respond well to anti-fungal treatment, limiting the progression of disease to stricture formation and necrotizing CE[72,73]. Therefore, although CE is rare, early detection and treatment of this condition in SSc esophagus is very important to avoid development of esophagus-related complications that have high mortality rates. Pill-induced esophagitis, which is characterized as direct injury to the esophageal mucosa due to use of certain culprit medications[74], is another important consideration in SSc patients. Interestingly, patient-related risk factors for pill-induced esophagitis are commonly associated with extended transit time of culprit medications in the esophagus. Studies suggest that altered anatomy, as found in SSc, can lead to delayed esophageal transit time and stasis, therefore increasing the risk for pill-induced esophagitis[74]. Other patient-related risk factors leading to increased transit time of medications include position of patient while taking pill and size of pill. Reduced water intake while ingesting pill, potentially due to impaired hand function in SSc also increases risk. Decreased saliva production secondary to Sjogren's syndrome, a condition known to have a higher prevalence in SSc patients, may also lead to dysphagia and impaired swallowing of the pill[74].

Notable culprit medications leading to pill-induced esophagitis include non-steroidal anti-inflammatory drugs, aspirin, bisphosphonates, potassium chloride, antibiotics (namely tetracycline and clindamycin), and iron. Prolonged contact of the pill with the esophageal mucosa can lead to direct irritation of the mucosa[75]. Several pathophysiologic mechanisms have been implicated in this condition including disruption of the cytoprotective prostaglandin barrier of the mucosa, caustic injury, and vascular injury resulting from hyperosmotic properties of the medication[74,76-78]. Pill-induced esophagitis often occurs at the site of esophageal narrowing which, in SSc patients, can result from stricture formation, decreased LES tone, or decreased esophageal peristalsis. This condition is typically self-limiting, but if left untreated, can lead to strictures, ulcerations, and even perforations, which can complicate dysphagia in SSc esophagus[79]. Ultimately, pill-induced esophagitis should be considered in SSc patients presenting with heartburn, dysphagia, or odynophagia and culprit medications should be avoided in this patient subset whenever possible. Special attention should be given to SSc patients that develop iron deficiency anemia, which often occurs secondary to microhemorrhages from telangiectasias in the GI mucosa and severe malabsorption along the GI tract in advanced disease[80]. Treatment with iron supplementation may be indicated, but usage of oral iron pills should be closely followed, as studies have shown that this can cause direct mucosal injury to the esophagus, leading to pill-induced esophagitis and dysphagia[81].

OTHER CONTRIBUTING FACTORS TO DYSPHAGIA

Gp

Gp is the delay of gastric emptying without mechanical obstruction, resulting in symptoms of bloating, nausea, and upper abdominal pain[82]. In SSc, Gp may develop due to fibrotic infiltration, resulting in subsequent dysfunction of autonomic nerves, smooth muscle, and enteric neurons[82]. Previous studies in SSc patients exhibiting GI symptoms have shown the prevalence of Gp to be 50%-67%[83]. GERD is commonly

associated with Gp, with about 27% of patients with Gp suffering from concomitant moderate to severe GERD[84]. The pathophysiology of this may be related to increased volume of gastric contents resulting in stomach distension and decreased LES pressure with more frequent TLESRs, causing regurgitation of contents into the esophagus[84]. This likely explains why GERD symptoms are found to be most severe in patients with Gp and other pre-existing conditions, such as SSc[3]. The complex interplay between Gp and severe GERD may be the reason why patients with SSc are found to have significantly greater reflux symptoms and complications than those with idiopathic reflux[51]. Correspondingly, treating GERD in patients with SSc may therefore involve treating the underlying Gp.

CONCLUSION

While it may be intuitive to blame esophageal dysmotility and reflux as the culprit for dysphagia in SSc patients, understanding the physiology of swallowing and anti-reflux defense mechanisms can provide better insight on causative and contributive factors in patient symptomatology. Understanding the range of potential sources for dysphagia, from oral to esophageal, can help develop more effective therapeutic plans that can improve symptoms and result in a better quality of life. Xerostomia may be managed through lifestyle changes, including drinking water frequently, using artificial saliva as needed, utilizing special toothpastes and mouthwashes, and avoiding medications that may exacerbate symptoms[2,85]. SSc patients should regularly perform exercises and massages to stretch their mouth and prevent debilitation from microstomia[2]. Regular follow-up with an experienced dentist can improve dental hygiene and oral health[2]. SSc patients presenting with dysphagia should also be screened for concurrent myositis, and treated accordingly with appropriate immunomodulatory therapy and other interventional procedures[37]. While studies show limited efficacy of treatment options targeting esophageal dysmotility, appropriate GERD management is essential in limiting the development of dysphagia[2]. GERD management includes dietary and lifestyle modifications as well as medication therapy to limit progression of reflux and reflux-related complications[2,57]. Delayed esophageal clearance may also be best managed through lifestyle changes such as avoiding meals before bedtime and elevating the head of the bed while sleeping[54]. Given that Candida infection and certain culprit medications can also cause esophagitis, providers should carefully screen for these in SSc patients. Signs of fungal infection should be treated promptly to prevent complications and culprit medications should be generally avoided[73,79].Underlying Gp may also contribute to symptoms and should be managed with prokinetic agents and dietary modification[2,57]. Being cognizant of the various contributing factors that result in dysphagia can allow physicians to develop better, more well-rounded therapeutic plans. This may allow better control of the various disease processes in SSc and improved symptoms and quality of life for SSc patients.

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ORIGINAL ARTICLE

Basic Study

Planning the hepatitis C virus elimination in Cyprus: A modeling study

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Abstract

BACKGROUND

Hepatitis C virus (HCV) infection is a major global public health problem. In the Republic of Cyprus, the estimated prevalence of chronic hepatitis C (CHC) among the general population is 0.6%, while the CHC prevalence among people who inject drugs (PWID) is estimated at 46%. Direct-acting antivirals that can eliminate HCV are not yet widely available in the Republic of Cyprus. However, when direct-acting antivirals become available, a long-term strategic plan to guide elimination efforts will be needed to maximize the effect of treatment.

AIM

To determine the programmatic targets to eliminate HCV in the Republic of Cyprus.

METHODS

A dynamic, stochastic, individual-based model of HCV transmission, disease progression, and cascade of care was calibrated to data from Cyprus. The model

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stratifies the population into the infected general population and the PWID population. A variety of test, prevention, and treatment strategies concerning the general population, PWID, or both were examined. The time horizon of the analysis was until 2034.

RESULTS

Under the status quo scenario, the model predicted that 75 (95% confidence interval (CI): 60, 91) and 575 (95%CI: 535, 615) liver-related deaths and new infections would occur by 2034, respectively. Launching an expanded treatment program, without screening interventions, would cause modest outcomes regarding CHC prevalence (16.6% reduction in 2034 compared to 2020) and liver-related deaths (10 deaths would be prevented compared to the status quo scenario by 2034). Implementing a test and treat strategy among the general population but without any intervention in the PWID population would suffice to meet the mortality target but not the incidence target. To achieve HCV elimination in Cyprus, 3080 (95%CI: 3000, 3200) HCV patients need to be diagnosed and treated by 2034 (2680 from the general population and 400 from PWID), and harm reduction coverage among PWID should be increased by 3% per year (from 25% in 2020 to 67% in 2034).

CONCLUSION

Elimination of HCV is a demanding public health strategy, which requires significant interventions both among the general population and high-risk groups.

Key Words: Mathematical modelling; Projections; Hepatitis C virus elimination; Direct-acting antivirals; Screening campaigns

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Core Tip: Direct-acting antivirals (DAAs) that can eliminate hepatitis C virus are not yet available in the Republic of Cyprus. However, when DAAs become available, a long-term strategic plan to guide elimination efforts will be needed to maximize the effect of treatment. To achieve the elimination goals, 3080 patients need to be diagnosed and treated by 2034 (2680 from the general population and 400 from people who inject drugs), and harm reduction coverage among people who inject drugs should be increased by 3% per year.

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INTRODUCTION

Hepatitis C virus (HCV) is a major public health problem that affects 1% of the world population[1]. The advent of highly effective direct-acting antivirals (DAAs) has significantly improved the management of the infection and brought great optimism that HCV could be eliminated in the near future[2,3]. Driven by the major clinical achievements, the World Health Organization (WHO) released the Global Health Sector Strategy on viral hepatitis targeting elimination by 2030[4]. The ambitious elimination targets include a 80% reduction in HCV incidence and a 65% reduction in HCV-related mortality in 2030 compared to 2015[4].

In the Republic of Cyprus (government-controlled area), the prevalence of chronic hepatitis C (CHC) among the general population is estimated at 0.6%, while the estimated CHC prevalence among people who inject drugs (PWID) is much higher at 43%[5]. Similar to other western countries, HCV transmission in the Republic of Cyprus is mostly limited to the PWID group[6].

Although DAAs are capable to eliminate HCV, they are not yet available in Cyprus. However, when DAAs become available, an appropriate long-term strategic plan to guide elimination efforts will be necessary to maximize the benefits of treatment. For example, to eliminate HCV is vital to implement screening campaigns among the general population to support treatment scale-up[7,8] and prevention/harm reduction (HR) measures among PWID to halt ongoing transmission[9-11].

Several studies from different countries have shown that the elimination of HCV is attainable using integrated strategies[7,8,12]. However, the WHO recommends countries develop country-specific targets within their national plans that align with their epidemiological situation. The optimal combination of the required intervention to achieve HCV elimination targets in Cyprus has not been studied yet. The aim of this study is to simulate the implementation of an integrated HCV strategy in the Republic of Cyprus to determine the programmatic targets to eliminate HCV.

MATERIALS AND METHODS

Setting

The HCV prevalence among adults in Cyprus ranges between 0.5%-1.9% with a central estimate of 0.6%[5,13]. Furthermore, according to the Cyprus National Addictions Authority, the estimated number of PWID in 2017 in Cyprus was between 499 and 909 (central estimate 700), of which, according to estimations, between 279 and 509 were HCV positive (central estimate 400) (Table 1).

The burden of HCV-related liver deaths is low since only 13 HCV-related deaths were recorded from 2005 to 2017 (2 HCV-related deaths in 2017)[5]. Regarding the fibrosis stage, only 15.7% of people living with HCV are estimated to have progressed to advanced disease (*i.e.* F3 Liver fibrosis stage and beyond) [5.2% and 10.5% are in F3 and ≥ F4, respectively (Supplementary Table 1)]. The main reason for that is that several patients with advanced disease had access to DAA treatment in the previous years individually either through the Cyprus' Committee of High-Cost Drugs or by ordering generic drugs from abroad.

Diagnosis efforts comprise a vital component of the elimination strategy of HCV. In the Republic of Cyprus, the diagnosis rate is moderate with 21 and 36 new diagnoses of CHC in 2017 and 2018, respectively[5]. However, 90 patients are currently on a waiting list to be approved for treatment with DAAs. Notably, DAAs will be available with no restrictions based on the fibrosis score.

HR strategies such as high-coverage needle and syringe programs and opioid substitution treatment have been often used to prevent the spread of HCV among PWID[14]. Several empirical studies have shown that these interventions can substantially reduce the risk of HCV acquisition among PWID[15]. In the Republic of Cyprus, the coverage of needle and syringe exchange programs is low, while the coverage of opiate substitution therapy is suboptimal because only about a quarter of PWID participate in any of them[16].

Description of the epidemiological model

A dynamic, discrete-time, stochastic, individual-based model of HCV transmission, disease progression, and cascade of care was fitted to epidemiological and clinical data from the Republic of Cyprus. The model stratified the population into two groups: infected general population (*e.g.*, HCV+ but not PWID) and the PWID population (Supplementary Figure 1).

The PWID population was stratified based on the following criteria: infection status (susceptible, infected), engagement in the HCV cascade care (undiagnosed, diagnosed, on DAAs), HR status (whether the PWID takes part in HR programs), and sharing status (sharer or non-sharer). PWID could transit from sharers to non-sharers and vice versa. However, the transition from sharers to non-sharers was balanced, so that the proportion of PWID in the high-risk group remained constant over time. Initially, new injectors do not participate in HR programs. Sexual transmission was not considered in the model.

The infected general population (*i.e.* infected patients without the risk to further transmit the disease) was divided by the fibrosis stage and the engagement in the HCV cascade of care (undiagnosed, diagnosed, on DAAs).

Each year, infected PWID exit the pool of injectors due to cessation of injection and transit to the infected general population. Individuals exit various states through HCV related death or background death (Supplementary Figure 1). PWID also experience additional drug-related mortality (Table 1).

Table 1 Model parameters and references

Parameter	Estimate	Ref.
Total chronic hepatitis C population size in the Republic of Cyprus	2900	[13]
Chronic hepatitis C population size in the Republic of Cyprus among the general population	2600	[13]
Chronic hepatitis C population size in the Republic of Cyprus among PWID	300	[16]
Proportion who are acutely infected and spontaneously clear infection	26%	[28]
PWID population	700	[16]
Duration of injecting carrier among PWID (yr)	13.5	[16]
Proportion of sharers	43%	[16]
Overall PWID mortality	2%	[29]
New diagnoses	30	[5]
Proportion participating in harm reduction programs (OST or high coverage HCNSP)	25%	[16]
Relative risk for HCV infection while in a harm reduction program	0.5	[14]
SVR IFN-free DAAs	95%	[17-19]
HCV Progression rates per yr		
F0→F1	0.176	[30]
F1→F2	0.082	[30]
F2→F3	0.100	[30]
F3→F4	0.161	[30]
F4→Decompensated cirrhosis	0.04	[31,32]
F4→Hepatocellular carcinoma	0.021	[31,32]
Decompensated cirrhosis→Death related to HCV	0.306	[31,32]
Hepatocellular carcinoma→Death related to HCV	0.433	[31,32]
Decompensated cirrhosis→Hepatocellular carcinoma	0.021	[31,32]

DAAs: Direct-acting antivirals; HCNSP: High-coverage needle and syringe programs; HCV: Hepatitis C virus; IFN: Interferon; OST: Opioid substitution treatment; PWID: People who inject drugs; SVR: Sustained virologic response.

We assume a 95% sustainable virologic response (SVR) rate for those treated with DAAs[17-19]. Individuals who have achieved SVR are considered cured. Patients who fail treatment could be retreated. If PWID achieve SVR, then they become susceptible again and are at risk of reinfection (assuming the risk of reinfection equals the initial infection rate and no behavior changes after successful treatment). Reinfected PWID could be retreated.

Infection probability

The force of infection for susceptible PWID depends on HCV prevalence and on whether they are high-risk, participating in HR programs, or both. More specifically, the force of infection for susceptible PWID who participate in an HR program is multiplied by a factor Z ($Z < 1$) indicating that PWID in HR programs have lower probability of getting infected compared to PWID not in HR programs. Low-risk PWID could not be infected (*e.g.*, PWID who do not share their injection equipment are not at risk of HCV infection).

Model calibration

The model was run until it achieved a steady-state (the level of prevalence in the population of PWID in 2020 without use of treatment) by varying the infection rate. After reaching a steady-state, the model was seeded with a cohort that represents the infected patients from the general population (size of the infected population, fibrosis stage, share of diagnosed, and mean age of the infected patients).

Intervention scenarios involving the expansion of testing and/or scaling-up treatment coverage and/or increased proportions of PWID in HR programs were examined. For each scenario, 1000 simulations (runs) were performed (Supplementary Figure 2). The results were summarized using the median of all simulations. To include the appropriate uncertainty (stochastic variability), credible intervals (*i.e.* 2.5 and 97.5 percentiles of simulations) are also shown. The time horizon of our analysis was 15 years (2020–2034). Further details about the description of the model are available in the appendix.

Scenarios analysis

Modified elimination targets: The WHO recommends each country develops country-specific targets aligned with its epidemiological situation. Several studies have highlighted the issue that for some countries the proposed WHO's elimination targets may be impractical[20,21]. For example, in countries like the Republic of Cyprus with low baseline mortality (approximately 2 per year), the 65% reduction may not be feasible.

The mortality target for the Republic of Cyprus was modified as follows: to prevent the cumulative number of deaths by 2034 from surpassing the limit of 5 per 100000 people (*e.g.*, 45 deaths) and to reduce CHC prevalence among the general population by over 80% in 2034 compared to 2020. Regarding the incidence goal, we kept the incidence target of the WHO (80% reduction in incidence in 2034 compared to 2020).

Examined scenarios

To examine the impact of different strategies on incidence, chronic prevalence, and HCV-related mortality, five different scenarios were considered.

Scenario A: A status quo scenario was used to generate predictions regarding the current management of HCV (Supplementary Figure 3). This provides a reference scenario of no regular scale-up of treatment (about 3 patients from the general population are individually treated per year). The additional four scenarios explored the impact of increased DAA uptake, HCV testing, and HR coverage.

Scenario B investigates the impact of increasing DAA coverage exclusively among the general population but without implementing awareness and screening programs (Supplementary Figure 4).

Scenario C is scenario B plus the implementation of awareness and screening programs among the general population (Supplementary Figure 5). This scenario explores the impact of an elimination strategy that exclusively focuses on the general population without any interventions among the high-risk population (*e.g.*, PWID population).

Scenario D is scenario C with increased HR coverage among PWID but without the simultaneous use of DAA therapy (Supplementary Figure 6). This scenario evaluates the impact of the expansion of primary prevention strategies among PWID without using DAA treatment. We assumed that PWID who participate in HR programs are diagnosed if they are infected and undiagnosed.

Scenario E is scenario D with the addition that DAA treatments would also be available to the PWID population (Supplementary Figures 7 and 8). This scenario assesses an integrated strategy that includes interventions both regarding the general population and the high-risk group.

Sensitivity analysis

To examine the impact of the uncertainty around epidemiological parameters or model assumptions in the required treatments to achieve elimination, a series of univariate sensitivity analyses under scenario E were implemented. More specifically the impact of higher/lower general population (1950 or 3250 *vs* 2600), higher/lower PWID population (525 or 875 *vs* 700), the impact of adherence to treatment on SVR (85% *vs* 95%), influence of shorter/longer average duration of injecting carrier (10 or 15 *vs* 13.5 years), and the effect of changes in risk behavior after successful treatment (50% lower or higher probability of re-infection *vs* no change in risk behavior).

RESULTS

Status quo scenario

Under the status quo scenario, the model predicted a 4.5% (95% confidence interval (CI): 3.2%, 5.7%) increase in the number of viremic cases in 2034 compared to 2020

(Figure 1A). Concerning liver-related deaths, the model projects that 75 (95%CI: 60, 91) liver-related deaths would occur between 2020 and 2034 under the status quo scenario (Figure 2).

Regarding HCV incident cases, it was estimated that 38 PWID (95%CI: 23, 51) are newly infected every year [annual incidence per 100 person-years: 9.6 (95%CI: 5.5, 13.2)] (Figure 3). Without any PWID-specific interventions, HCV incidence is expected to remain constant through our study. The cumulative HCV incident cases, under the status quo scenario, would be 575 (95%CI: 535, 615) during 2020-2034 (Supplementary Figure 10). Finally, the total number of PWID with CHC that would stop injecting and be considered part of the general population, under the status quo scenario, would be 340 (95%CI: 305, 360) during 2020-2034.

Scenario B

Under scenario B, 90 DAAs per year would be available for the infected population. However, without the implementation of awareness and screening interventions, the elimination program would quickly run out of patients after the first years due to the insufficient number of diagnosed patients (Figure 4B).

Scenario B would cause marginal declines in CHC prevalence and liver-related deaths. More specifically, the total number of infected people was projected to be lower by 16.6% (95%CI: 15.2%, 18.5%) in 2034 compared to 2020 (Figure 1A). Regarding deaths, scenario B would prevent 10 (95%CI: -12, 29) liver-related deaths compared to the status quo scenario by 2034. However, this decline would be insufficient to keep the number of deaths closer to the desired target (45 cumulative deaths) (Figure 3).

Finally, because in this scenario no antiviral treatments or expansion of HR coverage is provided to PWID, HCV incidence would remain constant (Figure 2).

Scenario C

Under scenario C, the modified targets of mortality could be achieved. More specifically, using a gradually increasing program, if we treat 2715 (95%CI: 2670, 2760) patients from the general population between 2020 and 2034, the prevalence of CHC in the general population will decrease by 82.7% (95%CI: 81.5%, 84.1%) in 2034 compared to 2020. Liver-related deaths will not exceed the limit of the 5 per 100000 deaths (Figures 1B, 3, and 4).

Scenario C would prevent 46 (95%CI: 27, 63) liver-related deaths compared to the status quo scenario (Figure 3). Nevertheless, no declines in incidence would be observed under this scenario (Figure 2).

Scenario D

Scenario D, in addition to scenario C, would prevent 59 (95%CI: 4, 120) new infections compared to the status quo scenario by 2034. However, without using DAAs for the PWID population, the estimated reduction in the incidence of HCV would be well below the WHO elimination goals (22.5% *vs* 80.0%) (Figure 2).

Furthermore, scenario D, compared to the previous scenarios, would reduce the transition of infected PWID, who stopped drug injection, to the general population. More specifically, expansion of HR would prevent 32 (95%CI: 9, 52) infections from passing from PWID to the general population.

Scenario E

The integrated strategy E can eliminate HCV in the Republic of Cyprus. To eliminate HCV in the country, HR coverage should be increased by 3% per year (from 25% in 2020 to 67% in 2034), and 3080 (95%CI: 3000, 3200) patients (2680 from the general population and 400 from PWID) should be diagnosed and treated with DAAs by 2034 (Figure 4, Supplementary Figure 9). To create the required DAA demand for the general HCV+ population, significant awareness and screening programs should be implemented. On the contrary, regarding the PWID population, because most of the PWID would be diagnosed through the expansion of HR coverage, there is no need for a significant screening program to identify the undiagnosed.

Under scenario E, the total number of viremic cases would decrease by 87.6% (95%CI: 86.4%, 88.7%) in 2034 compared to the number of viremic cases in 2020 (Figure 1A). Additionally, 45 (95%CI: 21, 68) and 175 (95%CI: 115, 220) liver-related deaths and new infections, respectively, would be averted compared to the status quo scenario by 2034 (Supplementary Figure 10).

The total number of chronic infected PWID that would cease injecting and move to the general population under scenario E would be 185 (95%CI: 160, 220) during 2020-

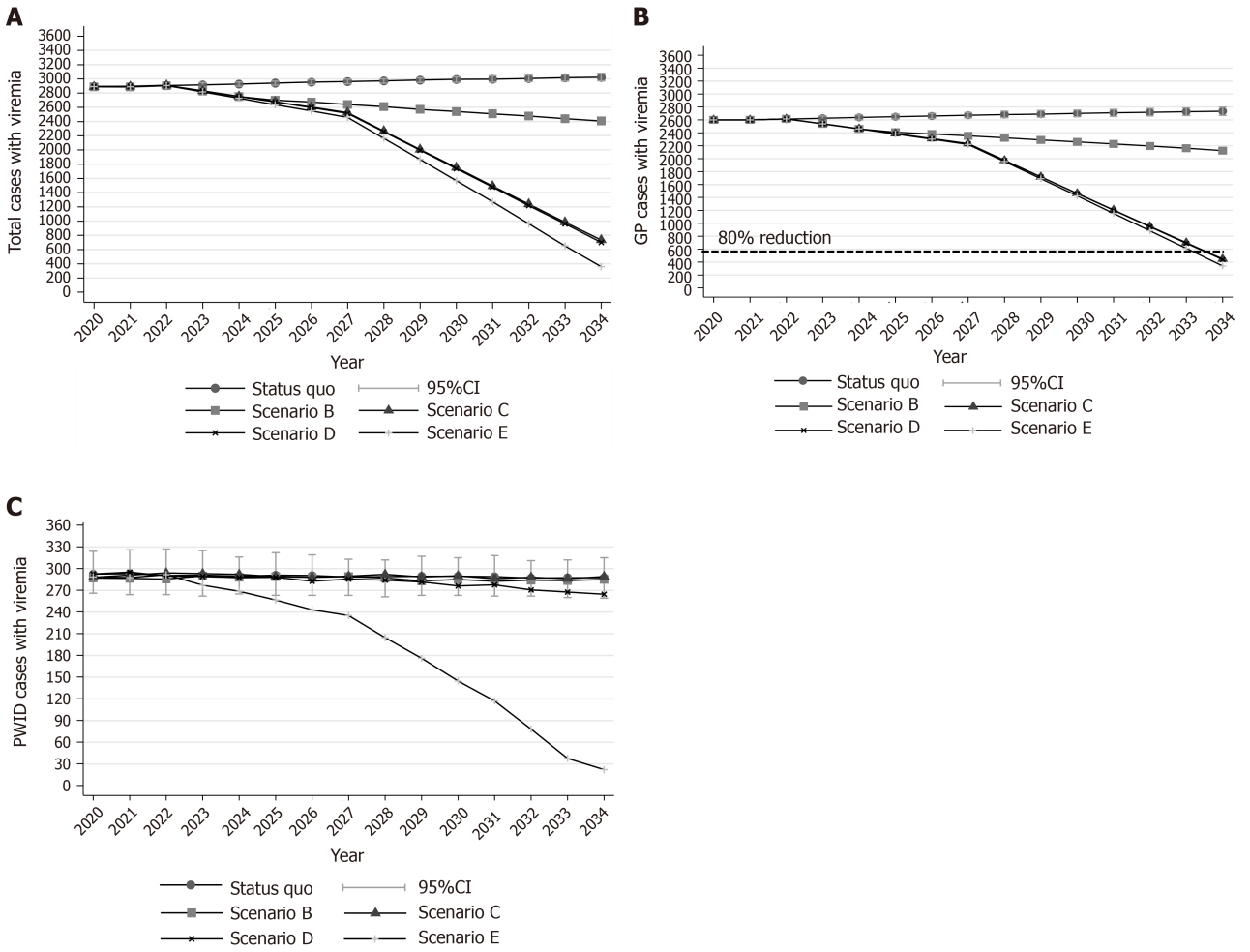


Figure 1 Model projections of future prevalence of chronic hepatitis C virus in the Republic of Cyprus under different scenarios for the total infected population, the general population (*i.e.* total infected population minus infected people who inject drugs), and the infected people who inject drugs. A: Total population; B: General population (GP); C: People who inject drugs (PWID). CI: Confidence interval.

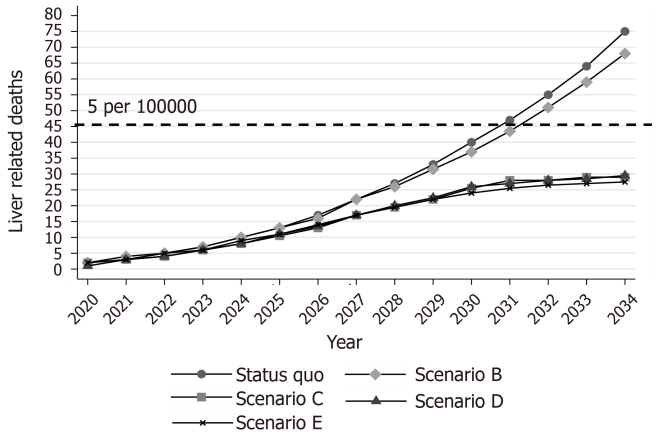


Figure 2 Model predictions for cumulative hepatitis C virus related deaths in the Republic of Cyprus under different scenarios.

2034.

Finally, under the elimination scenario, some reinfections among PWID would appear. Because there is no natural immunity following successful treatment, PWID with ongoing risk activities remain vulnerable to reinfection. It was estimated that 5 (95%CI: 1, 15) reinfections would occur during the horizon of the elimination strategy. (Supplementary Table 2).

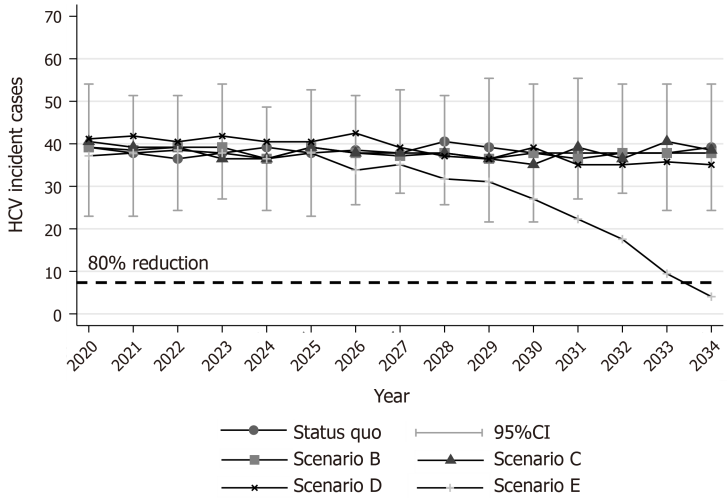


Figure 3 Model predictions for hepatitis C virus new cases (incidence) among people who inject drugs in the Republic of Cyprus under different scenarios. HCV: Hepatitis C virus.

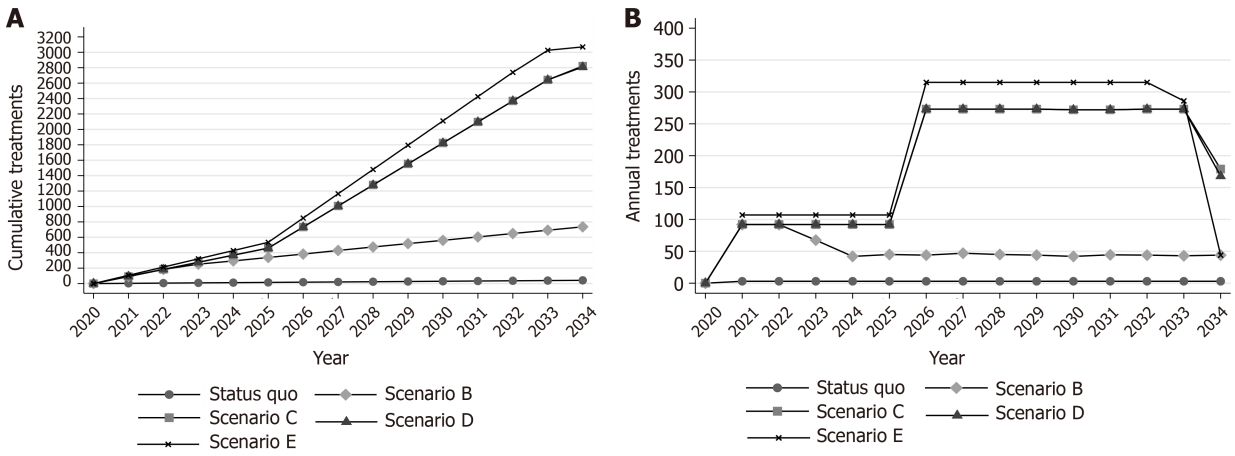


Figure 4 Model estimations concerning the cumulative and annual number of antiviral treatments of each scenario. A: Cumulative treatments; B: Annual treatments.

Sensitivity analysis

The sensitivity analysis showed that the variation in the size of the HCV+ general population is the primary determinant of the estimated required number of treatments in order to achieve HCV elimination in the Republic of Cyprus. Specifically, under a smaller population (*e.g.*, 1950 instead of 2600), the required treatments decreased by 19.3% compared to the base case. On the contrary, if the size of the HCV+ general population is higher than the base case (*e.g.*, 3250 instead of 2600), the needed treatment increased by 20.1%, compared to the base case (Supplementary Figure 11).

The second most important factor that affects the required number of treatments to achieve elimination is the variation in the average duration of drug use injection. For a longer injecting duration (15.0 years instead of 13.5 years), the needed treatments would increase by 3.6%. On the other hand, for a shorter injecting duration (10.0 years instead of 13.5 years) the required treatments would decrease by 3.4%. Lower SVR is expected to cause a 3.7% increase in the required number of treatments to achieve elimination.

Although most of the variables had a marginal impact on the projections regarding the elimination at the country level, they have a significant impact on the micro-elimination of CHC in PWID (Supplementary Figure 11). For example, potential changes in risk behavior of PWID after successful treatment is a vital factor regarding HCV elimination within the PWID population. If the risk of reinfection following treatment is 50% higher than the primary risk of infection (due to potential complacency caused by improvements in antiviral therapy), then the required number of treatments to reduce the incidence of HCV would 10% higher.

DISCUSSION

Our study is the first analysis that estimated the required interventions to achieve HCV elimination in the Republic of Cyprus. According to our model, HCV elimination in the Republic of Cyprus is feasible but necessitates significant improvements in the cascade of care, expansion in HR coverage, and scale-up of antiviral treatment. In this analysis, a mild gradual increase in DAA coverage was analyzed, as it is more realistic in settings where the majority of the infected population are undiagnosed and unlinked to care.

Because the elimination of HCV requires sustained and targeted efforts, well-defined targets are critical to guide action. Well-defined targets would help ensure enhanced HCV screening and committed funding to scaling-up treatment. The sustainable funding of the national action plan and the political will to achieve HCV elimination has been highlighted as key success factors for eliminating HCV as a public health threat[12].

Implementation of screening and linkage-to-care programs is a significant component of the elimination strategy[7,8,12]. To achieve the elimination targets, awareness activities should create enough demand for treatment. As scenario B illustrates, if awareness activities fail to diagnose enough patients, WHO elimination targets are unlikely to be reached because the elimination program would quickly run out of patients to treat (Figure 4B). This highlights the importance of taking a holistic approach to HCV elimination with coordinated screening, linkage-to-care, and treatment efforts. An HCV management without effective screening campaigns would be an ineffective health policy strategy[7,8].

Injection drug use remains the major risk factor for new HCV infections in the Republic of Cyprus. The efforts to eliminate HCV among PWID are a critical component of the country's elimination strategy. Elimination of HCV among PWID not only reduces new infections but also eliminates the number of infected ex-PWID who will enter the general population. For example, under the status quo scenario, the number of PWID who would acquire HCV and stop injecting is high: 340 (95%CI: 305, 360) infected ex-PWID by 2034. As most of those individuals are in an early phase of the disease and mostly without symptoms, it would be more difficult to be diagnosed and linked to care when they have been moved back to the general population. This suggests that if case finding interventions failed to identify infected PWID during their injecting carrier, intensifying screening campaigns in the general population to prevent HCV-related deaths attributed to the ex-PWID population should be implemented.

HR interventions have a vital and multifaceted role in reaching the incidence elimination target[9,22]. First, HR programs reduce both initial infections and reinfection after successful treatment and thus the required number of treatments to eliminate HCV. Second, HR services could play a potential case-finding role and serve as an access point to HCV education and counseling. It is important that if we expand HR coverage, the need for additional screening campaigns among PWID would be minimal (Supplementary Figure 8). Nevertheless, HR strategies must be provided for the long term, without any future discontinuation. Potential removal would allow the rebound of HCV infections, even after the elimination goals have been achieved[9,23]. Additionally, as scenario D highlights, if HR strategies are not coupled with antiviral therapy, the expected decreases in CHC prevalence and incidence would be limited.

Currently, HCV mortality is not considered a significant public health problem in Cyprus as there are few patients with advanced disease in the country. Nonetheless, our model highlights that complacency or inaction will cause a substantial number of preventable HCV-related deaths and HCV consequences (compensated cirrhosis, decompensated cirrhosis, and hepatocellular carcinoma) in the following years as a result of the aging of the HCV cohort.

Increases in reinfections may be a barrier to implementing treatment as prevention as an intervention to end transmission[24,25]. Reinfected cases are expected to exist during the elimination strategy because the susceptible population would increase without significant decreases in the infected population. Nevertheless, the existence of reinfections is an indirect indicator that we treat active PWID[9]. Sustained HCV treatment strategies will reduce the infected pool leading to the eventual reduction in the rate of HCV reinfection. In the Republic of Cyprus, reinfections are expected to be relatively low under scenario E due to the significant expansion of the HR coverage during the elimination strategy.

The implementation of the national elimination strategy is a dynamic procedure. Monitoring and evaluation of the progress of an elimination plan are essential to understand the effectiveness of the applied interventions and to determine the level of

improvement if needed. Although the strategy presented in our results could achieve the elimination of HCV, our mathematical model should be continuously rerun and fed with the most up-to-date data, so as to evaluate the achieved impact and keep the programs on track to achieve the elimination targets.

Added value of the study

Our study contributes to the discussion regarding the feasibility of HCV elimination. First, the analysis underlined that HCV elimination in Cyprus is achievable and computed the required interventions.

Second, due to the very low baseline HCV-related mortality (~2 per year), we have used both an absolute (*i.e.* preventing the cumulative number of deaths by 2034 from surpassing the limit of 5 per 100000 people) and a relative target (*i.e.* reducing CHC prevalence among the general population by over 80% in 2034 compared to 2020) regarding the mortality part of the elimination. Although the relative targets have been extensively used since their introduction, the absolute HCV elimination targets have recently been put on the agenda[20,21].

Comparisons with other studies

Our results are consistent with previous modeling studies showing that in order to meet the elimination targets, interventions to prevent transmission and increase testing simultaneously with the increase of treatment coverage should be implemented [11,22,26]. Furthermore, the outputs of our model are in line with other models showing that the impact of scaling-up treatment is significantly larger when combined with prevention programs[9-11,23]. Finally, our findings that the mortality target requires more treatment than needed to achieve the incidence target is consistent with previous studies[23,27].

Limitations

As with any modelling study, there are several limitations to our approach. First, the model ignores the impact of social networks on HCV transmission and assumes that the population is totally mixed, *i.e.* injectors have equal contact with all other injectors in the population. Second, we assumed that the proportion of sharers and non-sharers PWID remained constant over time after 2016. Third, the model did not take into account the impact of the coronavirus disease 2019 pandemic. Fourth, we assumed that the fibrosis distribution of the patients awaiting DAA therapy was similar to the whole HCV-infected population in the Republic of Cyprus. Finally, the model did not consider the additional mortality or potential increase of HCV progression rates due to HCV/human immunodeficiency virus coinfection.

CONCLUSION

The elimination of HCV is a demanding public health goal, which requires significant reforms. Our results show that the elimination of HCV cannot be achieved without implementing awareness and screening programs among the general population and prevention interventions among high-risk groups. Our model estimates that around 3000 patients need to be diagnosed and treated by 2034.

ARTICLE HIGHLIGHTS

Research background

Hepatitis C virus (HCV) infection is a major global public health problem. Although direct-acting antivirals are capable to eliminate HCV, they are not yet widely available in Cyprus. However, when direct-acting antivirals become available, an appropriate long-term strategic plan to guide elimination efforts will be necessary to maximize the benefits of treatment.

Research motivation

An appropriate long-term elimination plan will maximize the benefits of treatment.

Research objectives

This study aims to simulate the implementation of an integrated HCV strategy in the

Republic of Cyprus to determine the programmatic targets to eliminate HCV.

Research methods

A dynamic, discrete-time, stochastic, individual-based model of HCV transmission, disease progression, and a cascade of care was fitted to epidemiological and clinical data from the Republic of Cyprus. The model stratifies the population into two groups: the infected general population [*e.g.*, HCV+ but not people who inject drugs (PWID)] and the PWID population. The model was run until it achieved a steady-state (the level of prevalence in PWID population in 2020 without the use of a treatment) by varying the infection rate. After reaching a steady-state, the model was seeded with a cohort that represents the infected patients from the general population (size of the infected population, fibrosis stage, share of diagnosed, and mean age of the infected patients).

Research results

The analysis showed that under the status quo scenario 75 (95% confidence interval: 60, 91) and 575 (95% confidence interval: 535, 615) liver-related deaths and new infections would occur by 2034, respectively. Without screening interventions, launching an expanded treatment program would cause modest outcomes regarding chronic hepatitis C prevalence (16.6% reduction in 2034 compared to 2020) and liver-related deaths (10 deaths would be prevented compared to the status quo scenario by 2034). Implementing a test and treat strategy among the general population but without any intervention in the PWID population would suffice to meet the mortality target but not the incidence target. To achieve HCV elimination in Cyprus, 3080 (95% confidence interval: 3000, 3200) patients need to be diagnosed and treated by 2034 (2680 from the general population and 400 from PWID), and harm reduction coverage among PWID should be increased by 3% per year (from 25% in 2020 to 67% in 2034).

Research conclusions

Our study highlighted that without the implementation of large awareness or screening programs, HCV elimination cannot be achieved, due to suboptimal treatment coverage. Elimination of HCV is a demanding public health strategy that requires significant public health reforms (*e.g.*, enhancing harm reduction programs, implementing case-finding, linkage to care interventions).

Research perspectives

Elimination of HCV is a demanding public health intervention, which poses significant challenges in any health care system. Nevertheless, our analysis highlighted that HCV elimination is an achievable target.

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ORIGINAL ARTICLE

Clinical and Translational Research

Establishment and validation of a computer-assisted colonic polyp localization system based on deep learning

Sheng-Bing Zhao, Wei Yang, Shu-Ling Wang, Peng Pan, Run-Dong Wang, Xin Chang, Zhong-Qian Sun, Xing-Hui Fu, Hong Shang, Jian-Rong Wu, Li-Zhu Chen, Jia Chang, Pu Song, Ying-Lei Miao, Shui-Xiang He, Lin Miao, Hui-Qing Jiang, Wen Wang, Xia Yang, Yuan-Hang Dong, Han Lin, Yan Chen, Jie Gao, Qian-Qian Meng, Zhen-Dong Jin, Zhao-Shen Li, Yu Bai

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Abstract

BACKGROUND

Artificial intelligence in colonoscopy is an emerging field, and its application may help colonoscopists improve inspection quality and reduce the rate of missed polyps and adenomas. Several deep learning-based computer-assisted detection (CADe) techniques were established from small single-center datasets, and unrepresentative learning materials might confine their application and generalization in wide practice. Although CADes have been reported to identify polyps in colonoscopic images and videos in real time, their diagnostic performance deserves to be further validated in clinical practice.

AIM

To train and test a CADe based on multicenter high-quality images of polyps and preliminarily validate it in clinical colonoscopies.

METHODS

With high-quality screening and labeling from 55 qualified colonoscopists, a dataset consisting of over 71000 images from 20 centers was used to train and test a deep learning-based CADe. In addition, the real-time diagnostic performance of CADe was tested frame by frame in 47 unaltered full-ranged videos that contained 86 histologically confirmed polyps. Finally, we conducted a self-controlled observational study to validate the diagnostic performance of CADe in real-world colonoscopy with the main outcome measure of polyps per colonoscopy in Changhai Hospital.

RESULTS

The CADe was able to identify polyps in the test dataset with 95.0% sensitivity and 99.1% specificity. For colonoscopy videos, all 86 polyps were detected with 92.2% sensitivity and 93.6% specificity in frame-by-frame analysis. In the prospective validation, the sensitivity of CAD in identifying polyps was 98.4% (185/188). Folds, reflections of light and fecal fluid were the main causes of false positives in both the test dataset and clinical colonoscopies. Colonoscopists can detect more polyps (0.90 *vs* 0.82, $P < 0.001$) and adenomas (0.32 *vs* 0.30, $P = 0.045$) with the aid of CADe, particularly polyps < 5 mm and flat polyps (0.65 *vs* 0.57, $P < 0.001$; 0.74 *vs* 0.67, $P = 0.001$, respectively). However, high efficacy is not realized in colonoscopies with inadequate bowel preparation and withdrawal time ($P = 0.32$; $P = 0.16$, respectively).

CONCLUSION

CADe is feasible in the clinical setting and might help endoscopists detect more polyps and adenomas, and further confirmation is warranted.

Key Words: Computer-assisted detection; Artificial intelligence; Deep learning; Colonoscopy; Clinical validation; Colorectal polyp

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Core Tip: Our study indicated that the deep learning-based computer-assisted detection system trained from the dataset consisting of the largest number of polyps achieved high diagnostic performance on the test dataset of images, colonoscopy videos and clinical validation. This system might aid colonoscopists in finding more polyps and adenomas and deserves to be further validated in multicenter randomized trials.

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INTRODUCTION

Colorectal cancers (CRC) are the third most prevalent cancer and the second highest cause of cancer deaths worldwide[1]. Colonoscopy is considered to be the gold standard for CRC screening[2] but cannot detect all colonic neoplasms[3,4]. Colonoscopy has been reported to miss 17%-48% of adenomas, which are considered to represent the causes of 50%-60% of interval cancers[5-7].

To detect more polyps, a wealth of auxiliary devices and technology have been invented to improve adenoma detection rates (ADRs) and reduce adenoma miss rates (AMRs)[8,9]. However, these devices cannot overcome the limitations of colonoscopists themselves, who could overlook the polyps flashing across the video screen [10]. The ability of colonoscopists to detect adenoma is also influenced by alertness, fatigue and monitoring and varies greatly among individual colonoscopists[11,12]. In addition, even colonoscopists with a high ADR may still miss adenomas because they may become less vigilant about inspecting the remaining colon after detecting one adenoma (one and done effect)[13].

Over the last two decades, computer-assisted polyp detection has been increasingly explored to improve inspection quality and reduce AMR[4,14]. Recently, artificial intelligence has made remarkable breakthroughs in medical fields with deep learning and convolutional neural networks (CNNs)[15,16]. Deep learning automatically makes use of CNNs, which logically imitate the structure and activity of brain neurons, to learn detailed features of medical images[17]. With sufficient learning materials, CNNs can reach even greater real-time detection accuracy than human experts, which suggests that computer-assisted detection systems (CADes) might serve as real-time "experts" to improve the quality of colonoscopies[15,18-20]. However, despite promising findings, most current CADes were established using a limited number of colonoscopic images from a single center, which might limit the robustness and generalizability of findings in wide practice[18,21]. Therefore, we trained and tested the CNN-based CADe using colonoscopic images of multicenter datasets as well as tested and validated its diagnostic performance in real-time colonoscopy videos and real-world colonoscopy.

MATERIALS AND METHODS

Overview of study design

The colonoscopic records were retrospectively collected from 20 centers (Supplementary Table 1). Figure 1A illustrates the workflow of preprocessing images. Fifty-five qualified colonoscopists screened and labeled the images in a specific labeling system (Supplementary Video 1), and the labeled images were randomly divided into training and test datasets for the construction and testing of the CADe. The detailed preprocessing is illustrated in the Appendix A. To evaluate comprehensively ADe's real-time diagnostic performance, 47 unaltered full-range colonoscopy videos of routine practice, including 86 histologically confirmed polyps, were collected (Supplementary Table 2, Appendix B), and a self-controlled observational study was conducted for clinical validation in the Changhai Endoscopy Center.

Construction of CADe

The CADe was built based on the You Only Look Once v2 deep learning framework. The training dataset was used to build the model, and the testing dataset was used to test the performances with the details illustrated in the Appendix C.

Validation of the CADe in clinical colonoscopy

Figure 1B shows the process of enrolling patients. Consecutive outpatients aged 18-75 years who were scheduled for screening, surveillance and diagnostic colonoscopies were invited to participate in the validation between November 1, 2018 and December 10, 2018. Exclusion criteria included declined consent, age < 18 or > 75 years, poor

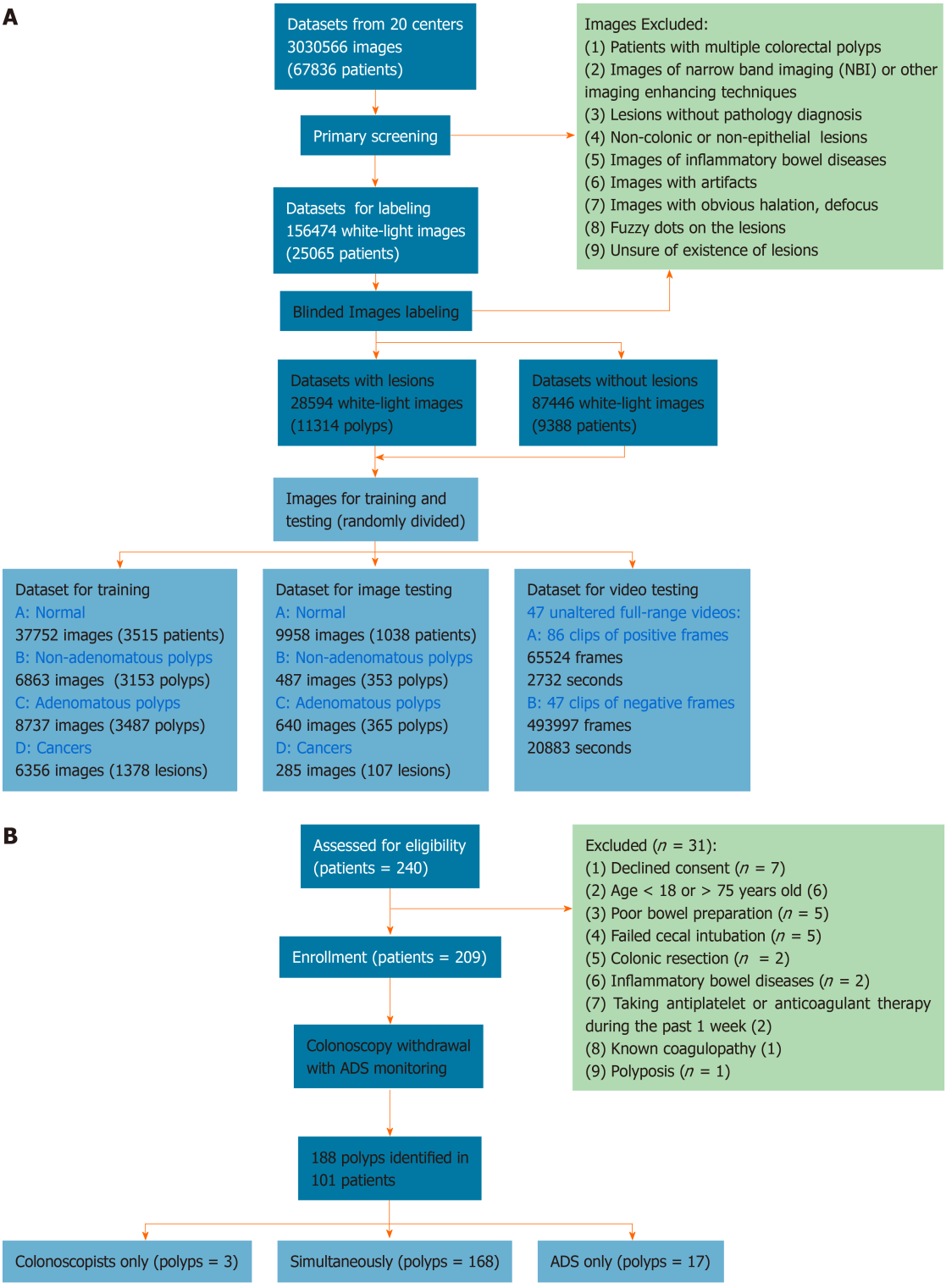


Figure 1 Flowchart of image preprocessing and validation in clinical trials. A: Image preprocessing; B: Validation in clinical trials.

bowel preparation quality, failed cecal intubation, history of colonic resection, inflammatory bowel disease, antiplatelet or anticoagulant therapy during the past 1 wk, known coagulopathy and polyposis. Patients received 3 L polyethylene glycol as a split-dose bowel preparation and were either sedated with propofol or without. Twenty colonoscopists, including two trainees, with experience ranging from 100 to over 20000 procedures performed colonoscopies, and an additional colonoscopist served as the full-time observer. The ADRs of the included colonoscopists ranged from 14%-33% in a mixed-indications population. Water-based colonoscopy, antispasmodics, distal attachments, optical-enhanced imaging and image-guided devices were not used in the validation.

CADe was automatically initiated when the ileocecal valve was identified. When identifying any potential polyp, the CADe presented an alert rectangle surrounding polyps on a second monitor and sounded alert, which was audible to colonoscopists and the observer. However, colonoscopists only observed the regular monitor of colonoscopy and were unable to see directly the alert rectangle since the second monitor (showing CADe's findings) was placed on their rear-right side. To view the findings identified by CADe, the colonoscopists had to turn back toward their right. When conducting withdrawal, the colonoscopists were required to offer immediately a verbal signal once they detected a potential polyp. The observer observed the two monitors, received alerts of CADe and signals from colonoscopists and finally determined the detection priority according to the following instructions.

Polyps identified in the study were finally confirmed by careful inspection of colonoscopists, biopsy and histologic examination, and the potential causes of false detection were determined by the discussion of colonoscopists and observers. Under the guidance of the observer, once hearing the alerts of the CADe, colonoscopists were required to recheck immediately all findings of the CADe, including observing the second monitor and carefully inspecting highlighted areas by the CADe. The duration of defining polyp-detection priority was confined to continuous withdrawal duration, and any process of observing the second monitor, seeking or unfolding polyps for rechecking, or performing biopsy and polypectomy was excluded. If the polyp was only identified by the CADe and colonoscopists could not localize the polyp before observing the second monitor, it was defined as type A (CADe only, missed by colonoscopists). If the polyp was reported by the colonoscopists and missed by the CADe before an unfolding or close view, it was defined as type B (colonoscopists only, missed by CADe). If the polyp was reported by both the colonoscopists and CADe during continuous withdrawal, it was defined as type C (colonoscopists and CADe). Therefore, types A + B + C included findings of the colonoscopists + CADe, whereas types B + C were classified as findings of the colonoscopists alone.

Outcome measures

For clinical validation, the main outcome measures were the mean polyps per colonoscopy and the sensitivity of CADe[22]. The mean adenomas per colonoscopy, false positives and negatives of CADe and potential causes were also analyzed. For the test dataset, the diagnostic performance was assessed based on the accuracy, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) with definitions illustrated in the Appendix D.

Sample size and statistical analysis

The sample estimation was based on the number of polyps per colonoscopy using the comparison of paired quantitative data. In the pilot trial, we found that the CADe could help increase the identification of 0.1 polyps per colonoscopy with a standard derivation of 0.37 for the improvement and assumed a power of 0.9 with an α-error of 0.01 to determine the sample size. Therefore, 203 patients were at least required for clinical validation. Continuous data are presented as the means and standard deviations. The number of polyps identified by each detection method was compared using a Wilcoxon signed ranks test. A 2-sided McNemar test with a significance level of 0.05 was performed to compare the polyp detection rate (PDR), ADR and sensitivity between colonoscopists and colonoscopists + CADe. A 2-sided P value < 0.05 was considered statistically significant, whereas P values for multiple groups were compared with relevant levels after Bonferroni correction. All statistical analyses were performed using SPSS Statistics v21 (IBM, Armonk, NY, United States).

Ethics and study registration

This study received approval from the ethics committee of Shanghai Changhai Hospital and registered at ClinicalTrials.gov (identifier, NCT03761771) and was performed in accordance with the Declaration of Helsinki. The use of retrospective data (images and historical reports) and the study protocol was approved or exempted by the ethics committee of every hospital. Informed and written consent was obtained from all patients during clinical validation, and patients had the right to withdraw at any time. All authors had access to the study data and approved the final manuscript.

RESULTS

Polyp identification in the test dataset

Supplementary Table 3 shows the characteristics of the polyps in the test dataset. There were 285, 640 and 487 images of adenocarcinomas, adenomatous polyps and nonadenomatous polyps in the test datasets, respectively (Supplementary Table 3). Of the 285 adenocarcinomas, 17.2% (49/285) could not be classified by the Paris classification because advanced stages were suspected. A total of 99.2% (483/487) of the nonadenomatous polyps and 96.6% (618/640) of the adenomatous polyps were classified as sessile or flat type (Supplementary Table 3).

The mean time for polyp identification of CADe was 0.03 ± 0.01 s. The receiver operating characteristic curve (ROC) is presented in Figure 2. Based on the ROC curve, the optimal cutoff value of probability was determined to be 50%, which is indicated by the red dot in Figure 2. The CADe shows excellent diagnostic performance in identifying polyps with an area under the curve of 0.984. Overall, the CADe identified colorectal lesions with 95.0% sensitivity, 99.1% specificity, 93.7% PPV and 99.3% NPV (Table 1), and the highest sensitivity was noted for adenocarcinomas (97.2%, Table 1). Moreover, CADe exhibited the highest sensitivity for large polyps (96.7% for ≥ 10 mm) and the lowest sensitivity for diminutive polyps (94.1% for ≤ 5 mm, Table 1). Fecal fluid/bubble (24%), reflection of light (21%), difficult angle (19%) and shadow (11%) were considered to be the four most common causes of false negatives (Table 2, Supplementary Figure 1), whereas folds (19%), reflections of light (18%), fecal fluid/bubble (17%) and colonic/ileocecal valves (19%) were the most important causes of false positives (Table 2, Supplementary Figure 2).

Polyp identification in colonoscopy videos

The video dataset consisted of 56 adenomas and 30 nonneoplastic polyps, and 53, 27 and 6 polyps exhibited diameters of ≤ 5 mm, 6-9 mm and ≥ 10 mm, respectively (Supplementary Table 2). Most of the included polyps (71/86) exhibited flat or sessile morphology, and a small portion of polyps (13/86) were considered challenging to detect in routine practice (Supplementary Table 2).

For frame-based tests, a dataset of 86 video clips of positive frames (65524 frames and 2732 s in total) and 47 clips of negative frames (493997 frames and 20883 s) were constructed for test. Although CADe identified all 86 polyps, regarding frame-based analysis, there was an overall sensitivity of 92.2% and specificity of 93.6% for overall polyps and a sensitivity of 66.2% and specificity of 97.9% for "challenging" polyps (Supplementary Table 4, Supplementary Video 2).

Validation of the CADe in clinical colonoscopy

In total, 240 patients were recruited for validation, and 31 were excluded due to refusal of informed consent (7), age (6), poor bowel preparation (5), failed cecal intubation (5) and others (8) (Figure 1B). The baseline characteristics are shown in Supplementary Table 3, and 188 polyps were identified in 101 patients (Figure 1B), resulting in a 48.3% PDR. The withdrawal time of 107 procedures (52.2%) was shorter than 6 min (Supplementary Table 5).

The number of polyps identified by colonoscopists only, CADe only and both were three, 17 and 168, respectively, revealing a sensitivity of 98.4% (185/188) for CADe (Supplementary Table 6). When assisted by the CADe, the number of identified polyps and adenomas significantly increased (0.82 *vs* 0.90, $P < 0.001$; 0.30 *vs* 0.32, $P < 0.05$, respectively), although the PDR and ADR did not increase ($P = 0.06$ and $P = 0.13$, respectively, Tables 3 and 4). The diagnostic sensitivity of the CADe was significantly greater than that of colonoscopists [98.4% (185/188) *vs* 91.0% (171/188), $P = 0.03$, Supplementary Table 6]. A total of 468 false positives occurred in 209 colonoscopy withdrawals (2.2 false positives per colonoscopy withdrawal); folds (59.0%), reflections of light (19.0%), fecal fluid/bubble (9.2%) and normal structures (9.8%) were considered to be the common causes of false positives (Table 2).

The diagnostic value of the CADe in polyps was observed in both age groups ($P = 0.03$ and $P = 0.002$, respectively), both sexes ($P = 0.005$ and $P = 0.01$, respectively), both hemi-colons ($P = 0.01$ and $P = 0.003$, respectively), all indications (all $P = 0.03$), inexperienced colonoscopists (< 1000 procedures) or general colonoscopists (1000-3000 procedures) ($P = 0.003$ and $P = 0.03$, respectively), and new generations of colonoscope (CF-290 and CF-260, $P < 0.001$ and $P = 0.03$, respectively), especially for diminutive (0.57 *vs* 0.65, $P < 0.001$) and flat polyps (0.67 *vs* 0.74, $P = 0.001$) (Table 3). Similarly, CADe also assisted colonoscopists in identifying more diminutive and flat adenomas ($P = 0.025$ and $P = 0.045$, respectively) in elderly (≥ 50 years, 0.43 *vs* 0.46, $P = 0.045$) and

Table 1 Performance of lesion detection and localization

	Lesions	Sensitivity, %	Specificity, %	PPV, %	NPV, %
Overall	1412	95.0 (93.7-96.0)	99.1 (98.9-99.3)	93.7 (92.3-94.9)	99.3 (99.1-99.4)
Pathology					
Non-adenomatous polyps	487	93.4 (90.8-95.4)	99.1 (98.9-99.3)	83.5 (80.0-86.5)	99.7 (99.5-99.8)
Adenomatous polyps	640	95.2 (93.1-96.6)	99.1 (98.9-99.3)	87.1 (84.4-89.5)	99.7 (99.5-99.8)
Adenocarcinomas	285	97.2 (94.3-98.7)	99.1 (98.9-99.3)	75.5 (70.7-79.7)	99.9 (99.8-100.0)
Size					
≤ 5 mm	713	94.1 (92.1-95.7)	99.1 (98.9-99.3)	88.2 (85.6-95.3)	99.6 (99.4-99.7)
6-9 mm	396	95.2 (92.5-97.0)	99.1 (98.9-99.3)	80.7 (76.8-84.1)	99.8 (99.7-99.9)
≥ 10 mm	303	96.7 (93.8-98.3)	99.1 (98.9-99.3)	76.5 (71.9-80.6)	99.9 (99.8-99.9)
Paris classification					
Ip	180	96.0 (91.8-98.3)	99.1 (98.9-99.3)	65.8 (59.7-71.4)	99.9 (99.8-1.00)
Is	581	96.1 (94.0-97.4)	99.1 (98.9-99.3)	86.1 (83.2-88.6)	99.8 (99.6-99.8)
IIa	525	92.6 (89.9-94.6)	99.1 (98.9-99.3)	84.4 (81.1-87.2)	99.6 (99.5-99.7)
IIb	30	100.0 (85.9-100.0)	99.1 (98.9-99.3)	25.0 (17.7-33.9)	100.0 (99.9-100.0)
IIc	2	100.0 (19.8-100.0)	99.1 (98.9-99.3)	2.2 (0.4-8.4)	100.0 (99.9-100.0)
III	32	96.9 (82.0-99.8)	99.1 (98.9-99.3)	25.6 (18.3-34.5)	100.0 (99.9-100.0)
Unclassified	62	96.8 (87.8-99.4)	99.1 (98.9-99.3)	40.0 (32.2-48.3)	100.0 (99.9-100.0)

PPV: Positive predictive value; NPV: Negative predictive value.

male patients (0.38 *vs* 0.41, $P < 0.001$), with the aid of a new-generation colonoscope (CF-290, $P = 0.025$) (Table 4). Notably, patients with adequate bowel preparation quality and withdrawal time showed a significantly increased number of polyps ($P < 0.001$ and $P = 0.001$) and adenomas ($P = 0.025$ and $P = 0.045$), whereas patients with < 6 Boston bowel preparation score and a < 6 min withdrawal time did not show any increase (Tables 3 and 4).

DISCUSSION

This CADe was developed and tested using over 70000 well-labeled colonoscopic images from 20 endoscopy centers, representing the dataset with the largest number of polyps. Notably, CADe did not merely perform well on the image tests of multicenter datasets and greater than 550 thousand frames of colonoscopy videos but also aided colonoscopists in identifying more polyps and adenomas in colonoscopy practice.

One of the first CADes systems validated in real-time colonoscopy was reported to identify polyps with 96% accuracy in the tests of labeled images[19], and consistently, the current CADe also reached a sensitivity of 95.0% for localizing polyps within a 30 ms constraint[23]. Notably, our findings further demonstrated that CADe assisted colonoscopists in detecting more adenomas in clinical practice but not merely in selected colonoscopy videos[19]. Compared with Yamada's CADe[24], the current CADe also realized a high sensitivity in the dataset mainly consisting of nonpolypoid lesions in contrast to Yamada's dataset, which was mainly based on polypoid lesions. However, the performance of the CADe seemed to decline in video tests (92.2% sensitivity and 93.6% specificity), which is consistent with previously reported CADes [20,24]. Further improvements in terms of process capability and colonoscopy video materials are warranted for CADes given that a great abundance and variety of random artifacts from the quick movement of videos with higher running speeds are indispensable to optimize further CADes in avoiding false positives and reducing time delays[19].

For clinical validation, several CADe systems in colonoscopy have been validated and are mainly focused on adenoma identification and quality control[25-31]. Wang *et*

Table 2 Potential causes of false negatives and false positives

Causes of false negatives	No. of images, *n* (%)	Causes of false positives[1]	No. of images, *n* (%)
Test dataset			
Fecal fluid/bubble	17 (24)	Folds	18 (19)
Reflections of light	15 (21)	Reflections of light	17 (18)
Difficult angle	13 (19)	Fecal fluid/bubble	16 (17)
Shadow	8 (11)	Colonic valve	11 (12)
Fuzzy image	6 (9)	Ileocecal valve	7 (7)
Color of background	2 (3)	Fuzzy image	5 (5)
Far distance	2 (3)	Others[2]	11 (12)
Unclassified	7 (10)	Unclassified	10 (11)
Validation in clinical colonoscopy			
Shadow	1 (33.3)	Folds[3]	276 (59.0)
Difficult angle	1 (33.3)	Reflections of light	89 (19.0)
Depressed lesion	1 (33.3)	Fecal fluid/bubble	43 (9.2)
		Normal structures[4]	46 (9.8)
		Other lesions[5]	14 (3.0)

[1]Twelve lesions were considered to be missed by senior colonoscopists on colonoscopy.
[2]Others included color change (3), anus (3), lesions under the mucous (2), melanosis coli (1), and bloodstain (1).
[3]Sixteen folds were caused by suction.
[4]Normal structures included vessels (20), ileocecal valve (12), anus (8), and lymphoid follicle (6).
[5]Other lesions included ulcers (7), cysts under the mucous (4), and diverticulum (3).

al[25,26,31] first conducted randomized controlled trials (RCTs) in an open-label or double-blind setting, most of which shared a large number of patients (both ~1000), rigorously illustrating that CADe effectively increased the ADR and adenomas per colonoscopy by identifying additional diminutive adenomas, including easy-to-miss adenomas for experienced colonoscopists and significantly reduced AMR[31]. Despite the demonstrated improvement of ADR and lower AMR in well-designed RCTs[25-31], our preliminary validation limited by the smaller sample size and observational design could not detect the difference in PDR or ADR and might calm our excessive excitement for AI. However, the observational and pragmatic design might reflect the realistic situation of colonoscopy practice and be beneficial for the real-world application of CADe. For example, in contrast to Wang's studies, which filtered out alerts of CADe when the lumen was not inflated, the colonoscopists in our validation directly received all alerts from CADe and could better evaluate the real-world feasibility of CADe and the level of colonoscopists' acceptance. We also analyzed all false positives rather than filtering out flashing-alert false positives given that the majority of the flashing alerts [folds (59.0%) and reflections of light (19.0%)] were clinically relevant to remind colonoscopists to inflate the lumen and carefully inspect the location of possibly missed polyps. Given that the exploration and application of CADe in colonoscopy is an emerging area and most studies were conducted in a single center, it is indispensable to explore prudently CADe's feasibility and endoscopists' acceptance in colonoscopy practice[32]. Presumably, the CADe should be further developed to identify different false positives to assess the bowel preparation quality and inspecting techniques as well as to remind the colonoscopist in real time to deal with the folds and residual fecal fluid, which may finally help to optimize high-quality colonoscopy.

Consistent with current work, Klare *et al*[29] also conducted an observational validation of their CADe with 55 clinical colonoscopies, indicating that their low-delay CADe might be feasible for real-time colonoscopy. However, their CADe detected no polyps before colonoscopists with a relatively greater false-positive frequency. Presumably, the construction of non-CNN algorithms and the design in which colonoscopists finally determine the criterion standard for polyp detection without rechecking the false positives of the CADe might lead to limitations[29]. Notably, our

Table 3 Polyp detection between colonoscopists and colonoscopists + computer-assisted detection

	Colonoscopists	Colonoscopists + CADe	*P* value
PDR, %	45.9	48.3	0.06
SSR, %	3.83	4.78	0.50
CRSR, %	9.09	11.5	0.06
Number of polyps, mean ± SD	0.82 ± 1.20	0.90 ± 1.25	< 0.001
Pathology			
Non-adenomatous	0.52 ± 0.94	0.58 ± 1.00	0.01
Adenomatous	0.30 ± 0.62	0.32 ± 0.64	0.025
Age			
< 50	0.45 ± 0.70	0.51 ± 0.77	0.03
≥ 50	1.12 ± 1.42	1.22 ± 1.47	0.002
Sex			
Male	0.96 ± 1.28	1.05 ± 1.36	0.005
Female	0.66 ± 1.07	0.72 ± 1.11	0.01
Location			
Proximal	0.35 ± 0.69	0.39 ± 0.76	0.01
Distal	0.46 ± 0.89	0.51 ± 0.90	0.003
Size			
≥ 10 mm	0.06 ± 0.28	0.06 ± 0.28	1
6-9 mm	0.18 ± 0.52	0.19 ± 0.52	0.32
≤ 5 mm	0.57 ± 0.95	0.65 ± 1.00	< 0.001
Morphology			
Flat	0.67 ± 1.03	0.74 ± 1.08	0.001
Subpedunculated	0.11 ± 0.42	0.12 ± 0.42	0.16
Pedunculated	0.03 ± 0.21	0.03 ± 0.21	1
Indications			
Screening	0.76 ± 1.15	0.85 ± 1.21	0.03
Surveillance	1.19 ± 1.42	1.33 ± 1.53	0.03
Diagnosis	0.70 ± 1.10	0.75 ± 1.13	0.03
Colonoscopes			
CF-Q290	0.78 ± 1.09	0.87 ± 1.17	< 0.001
CF-Q260	1.04 ± 1.58	1.15 ± 1.56	0.08
CF-Q240	0.83 ± 1.64	0.83 ± 1.64	1
Experience			
> 3000	1.24 ± 1.48	1.24 ± 1.48	1
1000-3000	0.79 ± 1.16	0.91 ± 1.26	0.003
< 1000	0.54 ± 0.92	0.62 ± 0.99	0.03
BBPS			
< 6	0.59 ± 1.19	0.62 ± 1.19	0.32
≥ 6	0.87 ± 1.19	0.96 ± 1.26	< 0.001
Withdrawal time			
< 6 min	0.52 ± 0.97	0.54 ± 0.98	0.16

≥ 6 min	1.13 ± 1.33	1.27 ± 1.39	0.001

CADe: Computer-assisted detection; BBPS: Boston bowel preparation score; PDR: Polyp detection rate; SSR: Sessile serrated adenoma/polyp detection rate; CRSR: Clinical serrated polyp detection rate.

findings indicated that the diagnostic capability of CADe tended to be susceptible to inadequate withdrawal time or inadequate bowel preparation quality (P = 0.32 and P = 0.16) given that CADe could only detect polyps appearing in view during the colonoscopy but was unable to identify polyps covered by fecal fluid or hidden between the folds. Therefore, the bowel preparation and withdrawal time should also be considered even if the CADe is established to monitor colonoscopy withdrawal. The combination of an AI-assisted quality control system (monitoring withdrawal speed and bowel preparation quality) and CADe could provide more remarkable benefits in adenoma detection, even for large adenomas[28]. However, the additional detection of most CADes, including our current system, is confined to nonadenomatous polyps and diminutive adenomas, of which clinical significance was undetermined for interval cancers and cost-effectiveness analysis. Although promising preliminary findings seemed to appear, the CADes should be further validated in multicenter settings and community practices as well as optimized for satisfactory integration into clinical practice.

We defined a true positive with intersection over union (IoU) > 0.3, which was also adopted in previous reports[33]. In addition, Horie *et al*[34] considered the detection of CADe correct when the CADe could recognize merely a part of the esophageal cancer and false when identifying wide noncancerous areas occupying greater than 80% of the frame, whose standard was significantly lower than the level of 0.3 in IoU. In addition, Paul *et al*[33] proposed to use > 0.3 IoU in training and > 0.1 IoU to distinguish pulmonary nodules with the explanation of respecting the clinical need for coarse localization. However, there was no uniform standard for IoU, and more studies are warranted to discuss the optimal IoU or define other potential indicators to ensure the quality and comparability between CADes. Given that colonoscopists in our study were aware that their examinations were being monitored and reminded by sound of the CADe, the result might be vulnerable to the Hawthorne effect, which may explain our study's significantly higher PDR (45.9%) compared to our previous report (PDR: 32.0-39.8%). Presumably, a fair proportion of polyps first detected by CADe or detected simultaneously by CADe and colonoscopists, which might have been ignored in real clinical practice, were additionally identified by colonoscopists in the current study. Although this weakness in our design may have prevented the benefit of CADe from being completely realized, more polyps and adenomas, especially those that were diminutive (< 5 mm) and flat, were still identified with the aid of CADe in the current study.

Compared with previous studies, our study has several strengths. First, our study retrospectively collected the datasets of 20 endoscopy centers, carefully screened patients with one polyp as well as the corresponding histological results, and deliberately labeled the images with blinding and repetition, finally constructing a large and high-quality dataset of colonoscopic images for training and testing. As a result, the dataset could represent a wider population and be ensured of the higher reliability of the original material, which is beneficial to reduce the risk of overfitting. Second, through pilot validation in clinical colonoscopy, our study not only indicated that CADe could help colonoscopists identify more polyps and adenomas but also preliminarily indicated the significance of quality control of colonoscopy (withdrawal time and bowel preparation quality) in CADe. Third, we analyzed the performance of CADe in various clinical scenarios and identified the most important causes of false negatives and positives, which might provide directions to optimize further the clinical application of the CADe.

Our study had several limitations. First, the CADe was completely established on selected retrospective white-light images without making use of the temporal coherence of videos, which contain many unclear and unfocused frames of images that could be not or mistakenly detected by CADe. As a result, the performance of CADe might be suboptimal with lower sensitivity or specificity in colonoscopy videos. Consistent with the concerns, the CADe's performance was influenced by the unclear frames (too distant, unfocused and partially appeared) of seven challenging videos, which led to a testing sensitivity of less than 80%. Further efforts should be made to train the CADe with more consecutive videos to take advantage of the temporal coherence and improve diagnostic accuracy in clinical colonoscopy. Second, we did

Table 4 Adenoma detection between colonoscopists and colonoscopists + computer-assisted detection

	Colonoscopists	Colonoscopists + CADe	*P* value
ADR, %	22.0	23.9	0.13
Number of adenomas, mean ± SD			
	0.30 ± 0.62	0.32 ± 0.64	0.025
Age			
< 50	0.14 ± 0.40	0.15 ± 0.41	0.32
≥ 50	0.43 ± 0.67	0.46 ± 0.69	0.045
Sex			
Male	0.38 ± 0.70	0.41 ± 0.73	0.045
Female	0.21 ± 0.48	0.22 ± 0.48	0.32
Location			
Proximal	0.12 ± 0.38	0.13 ± 0.39	0.16
Distal	0.17 ± 0.46	0.19 ± 0.47	0.08
Size			
≥ 10 mm	0.06 ± 0.28	0.06 ± 0.28	1
6-9 mm	0.11 ± 0.36	0.11 ± 0.36	1
≤ 5 mm	0.12 ± 0.38	0.15 ± 0.42	0.025
Morphology			
Flat	0.20 ± 0.47	0.21 ± 0.50	0.045
Subpedunculated	0.07 ± 0.27	0.07 ± 0.28	0.32
Pedunculated	0.03 ± 0.21	0.03 ± 0.21	1
Indications			
Screening	0.29 ± 0.65	0.33 ± 0.66	0.08
Surveillance	0.43 ± 0.67	0.45 ± 0.67	0.32
Diagnosis	0.25 ± 0.56	0.26 ± 0.59	0.32
Colonoscopes			
CF-Q290	0.31 ± 0.63	0.34 ± 0.65	0.025
CF-Q260	0.23 ± 0.51	0.23 ± 0.51	1
CF-Q240	0.25 ± 0.62	0.25 ± 0.62	1
Experience			
> 3000	0.5 ± 0.72	0.5 ± 0.72	1
1000-3000	0.24 ± 0.59	0.27 ± 0.60	0.08
< 1000	0.23 ± 0.53	0.26 ± 0.60	0.16
BBPS			
< 6	0.19 ± 0.46	0.19 ± 0.46	1
≥ 6	0.32 ± 0.64	0.35 ± 0.66	0.025
Withdrawal time			
< 6 min	0.22 ± 0.57	0.22 ± 0.60	0.32
≥ 6 min	0.38 ± 0.65	0.42 ± 0.65	0.045

CADe: Computer-assisted detection; BBPS: Boston bowel preparation score; ADR: Adenoma detection rate.

not identify a significant difference in PDR and ADR due to a limited sample size

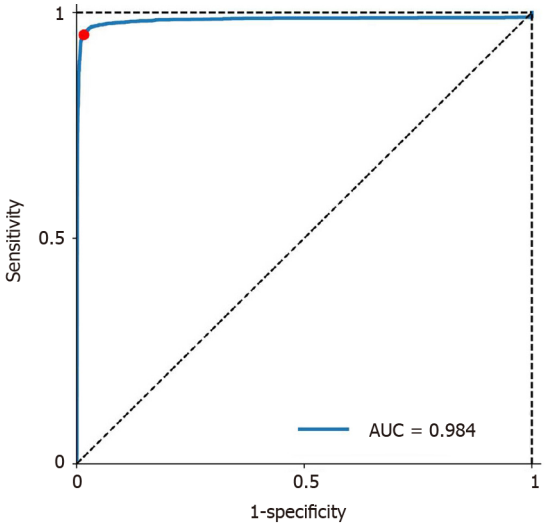


Figure 2 Receiver operating characteristic curve for localization of polyps. AUC: Area under the curve.

despite the fact that the polyps and adenomas identified per colonoscopy significantly increased. This information might provide a potential remedy to missed adenomas and the one-and-done effect of colonoscopists, which was demonstrated in recent tandem RCTs[31]. Finally, we artificially defined and classified the detection of polyps into three types. The validity of this classification was uncertain and might be influenced by the investigator's bias (*i.e.* the delay between the recording time and actual time of detection on the part of the colonoscopists) despite the independent determination of observer using the objective measurement of time. As a result, the current study might not be completely adequate to demonstrate the clinical implementation of CADe due to the single-center setting with a limited sample size, and future large RCTs are warranted.

CONCLUSION

In summary, our study indicated that this deep learning-based CADe had been trained on the dataset consisting of the largest number of polyps from 20 centers and reached high diagnostic performance on the test dataset, colonoscopy videos and clinical validation with an acceptable frequency of false positives. This system might help colonoscopists identify more polyps and adenomas and deserves to be further validated in multicenter randomized trials.

ARTICLE HIGHLIGHTS

Research background

Artificial intelligence is an emerging area in the research and applications of digestive endoscopy. Current deep learning-based computer-assisted detection (CADe) for colorectal polyps is mainly constructed from single-center and limited-sample learning images.

Research motivation

Although several studies reported that CADes identified polyps in colonoscopic images, videos and exams in real time, the lack of large-sample and representative learning materials might limit the diagnostic performance in wide colonoscopy practice.

Research objectives

We aimed to train and test a CADe based on a multicenter high-quality dataset of polyps and preliminarily validated it in clinical colonoscopies.

Research methods

After retrospective collection and systematic screening and labeling, over 71000 images from 20 centers and 47 unaltered full-ranged videos were used to train and test a deep learning-based CADe. We also validated its diagnostic performance in real-world colonoscopy using a single-arm self-controlled observational study.

Research results

Our CADe identified polyps of images with 95.0% sensitivity and 99.1% specificity. For colonoscopy videos, all 86 polyps were detected with 92.2% sensitivity and 93.6% specificity for frame-by-frame analysis. The sensitivity of CAD in identifying polyps was 98.4% (185/188) in the prospective validation. Colonoscopists could detect more polyps (0.90 *vs* 0.82, $P < 0.001$) and adenomas (0.32 *vs* 0.30, $P = 0.045$) with the aid of CADe, particularly polyps < 5 mm and flat polyps/adenomas.

Research conclusions

A CADe based on multicenter high-quality colonoscopic images was constructed and might help endoscopists detect more polyps and adenomas.

Research perspectives

CADe is feasible in the clinical setting, and further confirmation is warranted.

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ORIGINAL ARTICLE

Case Control Study

Tailored eradication strategy *vs* concomitant therapy for *Helicobacter pylori* eradication treatment in Korean patients

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Abstract

BACKGROUND

Antibiotic resistance to *Helicobacter pylori (H. pylori)* infection, which ultimately results in eradication failure, has been an emerging issue in the clinical field. Recently, to overcome this problem, an antibiotic sensitivity-based tailored therapy (TT) for *H. pylori* infection has received attention.

AIM

To investigate the efficacy and safety profiles of TT for *H. pylori* infection treatment compared to a non-bismuth quadruple therapy, concomitant therapy (CT) regimen.

METHODS

We included patients (> 18 years) with an *H. pylori* infection and without a history of *Helicobacter* eradication who visited the Gil Medical Center between March 2016 and October 2020. After being randomly assigned to either the TT or CT treatment group in 1 to 1 manner, patient compliance, eradication success rate (ESR), and patient-reported side effects profiles were assessed and compared between the two groups. *H. pylori* infection was diagnosed using a rapid urease test, Giemsa stain, or dual priming oligonucleotide polymerase chain reaction (DPO-PCR). Tailored eradication strategy based through the presence of a 23S ribosomal RNA point mutation. For the TT group, a DPO-PCR test, which detected A2142G

study protocol was approved by the ethics committee of the GMC.

Informed consent statement: Patients were not required to give the informed consent to the study because the analysis used the anonymous data that were collected after each patient agreed to treatment.

Conflict-of-interest statement: All the Authors have no conflict of interest related to the manuscript.

Data sharing statement: The data used to support the findings of this study are available from the corresponding author upon request at (junwonchung@gilhospital.com)

STROBE statement: The authors have read the STROBE Statement—checklist of items, and the manuscript was prepared and revised according to the STROBE Statement—checklist of items.

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and/or A2143G point mutations, and a clarithromycin resistance test were performed. Patients in the clarithromycin-resistant group were treated with a bismuth-containing quadruple combination therapy, while those with sensitive results were treated with the standard triple regimen.

RESULTS

Of the 217 patients with a treatment naive *H. pylori* infection, 110 patients [mean age: 58.66 ± 13.03, men, n = 55 (50%)] were treated with TT, and 107 patients [mean age: 56.67 ± 10.88, men, n = 52 (48.60%)] were treated with CT. The compliance (TT *vs* CT, 100% *vs* 98.13%, P = 0.30), and follow-up loss rates (8.18% *vs* 9.35%, P = 0.95) were not significantly different between the groups. The ESR after treatment was also not statistically different between the groups (TT *vs* CT, 82.73% *vs* 82.24%, P = 0.95). However, the treatment-related and patient-reported side effects were significantly lower in the TT group than in the CT group (22.77% *vs* 50.52%, P < 0.001).

CONCLUSION

The DPO-based TT regimen shows promising results in efficacy and safety profiles as a first-line *Helicobacter* eradication regimen in Korea, especially when physicians are confronted with increased antibiotic resistance rates.

Key Words: *Helicobacter pylori*; Eradication; Tailored therapy; Conco-mitant therapy regimen

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Core Tip: We investigated the efficacy and safety profiles of a tailored therapy (TT) as a first line *Helicobacter pylori* (*H. pylori*) eradication treatment compared to a concomitant therapy (CT) regimen in Korea, where clarithromycin resistance rates are high. Of 217 treatment-naïve *H. pylori* infection patients, 107 patients were treated with CT and 101 patients with TT. Although the eradication success rate was not statistically different between the groups, the treatment-related side effect rate was significantly lower in the TT group. Therefore, the TT regimen might be a promising solution to overcoming the problem of increased antibiotic resistance rates for *Helicobacter* eradication.

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INTRODUCTION

Helicobacter pylori (H. pylori) eradication is associated with a reduced risk of stomach cancer, including adenocarcinoma and mucosa-associated lymphoid tissue lymphoma, thus the rising global rates of antibiotic resistant *H. pylori* infections is concerning[1-4]. Currently, the Maastricht V/Florence Consensus guidelines, and Korean *H. pylori* treatment guidelines recommend proton pump inhibitor-based standard triple therapy [5-7]. However, the treatment success rate of standard tailored therapy (TT) has been declining worldwide over the past few decades as *H. pylori* drug resistance has increased year-over-year[4,7-9]. In Korea, a recent studying assessing the use of the standard triple therapy regimen for *H. pylori* eradication showed a treatment success rate of less than 70%[8-11]. Given that the ideal eradication success rate (ESR) should be over 80% in intention-to-treatment (ITT) analyses, and 90% in per-protocol (PP) analyses according to the 1997 Asia-Pacific Agreement Report for *H. pylori* treatment, conventional standard triple therapy with an empirically chosen policy has lost its role in *H. pylori* eradication practice[12].

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Recently, to overcome the aforementioned problems of empirically chosen eradication policies, the concept of 'TT' has been introduced in the eradication policy for *H. pylori* infection[12-18]. TT for *H. pylori* eradication is based on a pre-treatment antibiotic resistance test using stool or stomach biopsy samples[18,19]. However, tissue culture based antibiotic resistance testing for *H. pylori* is not ideal in that it is costly, time consuming, and not all of the antibiotics used in the regimen can be tested. Instead, dual priming oligonucleotide polymerase chain reaction (DPO-PCR) has been used[20-22]. DPO-PCR tests are cost effective and less time consuming than tissue culture based tests[23,24]. However, DPO-PCR test is currently only available for clarithromycin (CLR) resistance testing, as a method for rapid metronidazole (MTZ) resistance testing for *H. pylori* has been invented clinically. However, Korea is a region with high CLR resistance. In fact, the resistance rate has gradually increased, from 22.9% in 2003–2005 to 37.0% in 2007–2009, with the major barriers for *H. pylori* eradication success being CLR resistance, prompting clinical data to be accumulated using DPO-PCR tests in *H. pylori* eradication regimens[9,25].

Unfortunately, there is little data on the efficacy and safety profiles of the TT regimen compared to the concomitant therapy (CT) regimen in first-line *H. pylori* eradication.

Herein, we investigated the efficacy (ESR) and safety profiles (treatment-related side effect events) of the TT regimen as compared to those of CT in patients with treatment-naïve *H. pylori* infections in Korea, where the CLR resistance rate is high (> 15%).

MATERIALS AND METHODS

Institutional review board approval

The Institutional Review Board of the Gil Medical Center (GMC) reviewed the study protocol and ethics. This study was conducted in accordance with the Declaration of Helsinki, and the study protocol was approved by the ethics committee of the GMC.

Enrolled study population

We enrolled patients (> 18 years) with evidence of an *H. pylori* infection who were treatment naïve for *H. pylori* eradication that visited the GMC (Incheon, Korea) between March 2016 and October 2020. Exclusion criteria for this study were as follows: (1) Patients under 18 years of age; (2) Patients with a history of previous eradication; (3) Patients with a history of an allergy to any medication used in this study; (4) Patients with any operation history regarding the stomach; (5) Seriously ill patients with critical medical history [heart failure (≥ New York Heart Association class II), severe respiratory illness, decompensated liver cirrhosis, terminal or supportive care stage of malignancy, *etc.*]; (6) Patients who could not afford to revisit the hospital for follow-up after medication; and (7) Patients who could not take medication orally.

Enrolled patients were randomly assigned to either the TT group or the CT group in a 1 to 1 manner and their compliance rates, ESRs, and treatment-related side effect rates were assessed (Figure 1).

Diagnosis of H. pylori infection

All patients underwent an upper gastrointestinal endoscopy and showed positive results through a rapid urease test, Giemsa stain, or DPO-PCR.

PCR guided CLR resistance test for TT group

We used DPO-PCR to detect the presence of either a 2142G or 2143G point mutation on 23S rRNA. The point mutation in the V domain of 23S rRNA has been depicted as a major risk factor for CLR resistance against *H. pylori*, and 2142G and 2143G are the most frequent sites for 23S rRNA point mutations. The wild type for both 2142G and 2143 G mutations was defined as CLR sensitive.

Eradication regimen for TT group, and CT group

For the TT group, after the DPO-PCR test, patients who were deemed CLR resistant were administered a bismuth-containing quadruple combination (PBMT), and those who were CLR sensitive with a standard pancreatic adenocarcinoma (PAC) regimen (Figure 1). The bismuth-containing quadruple regimen consisted of 30 mg of lansoprazole twice daily + 500 mg MTZ twice daily + 300 mg bismuthate four times daily + 500 mg tetracycline four times daily for 10 d. The PAC regimen consisted of 30

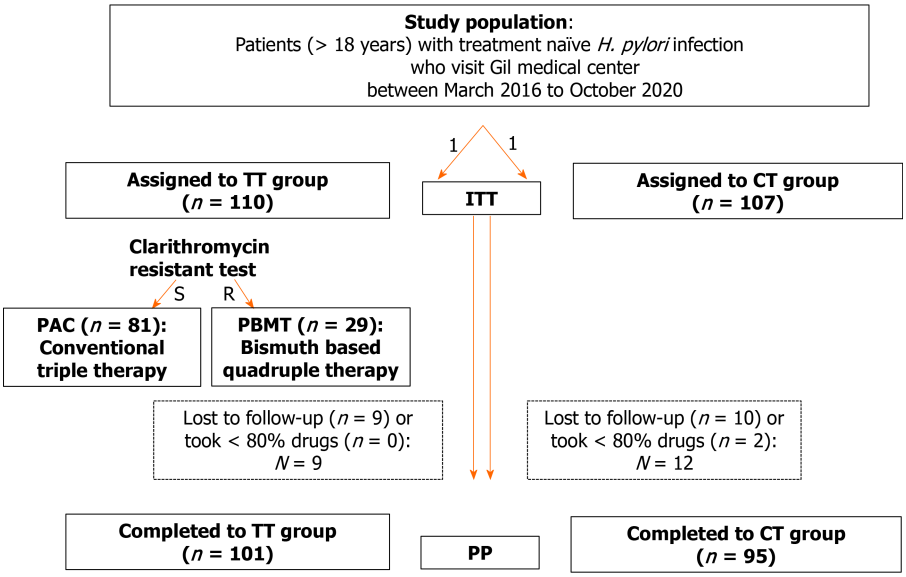


Figure 1 Flow chart. TT: Tailored therapy; CT: Concomitant regimen; S: Sensitive; R: Resistance; PAC: Pancreatic adenocarcinoma; PBMT: Bismuth-containing quadruple combination; ITT: Intention-to-treat; PP: Per-protocol; *H. pylori*: *Helicobacter pylori*.

mg lansoprazole + 500 mg CLR + 1000 mg amoxicillin (AMX), administered twice daily for 14 d.

The CT regimen consisted of 30 mg lansoprazole twice a day + 1 g AMX twice a day + 500 mg MTZ twice a day + 500 mg CLR twice a day for 10 d.

Follow up strategy, and outcome interpretation (efficacy and safety profiles)

Four weeks after finishing their eradication medication, patients were recommended to visit the GMC and undergo the ^{13}C-ure breath test (UBT; UBiTkit; Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan) with a cut-off value of delta $^{13}CO2 < 2.5‰$ to evaluate eradication success. During this visit, the patients reported treatment-related side effects and drug compliance rates were also recorded by the physician.

Definition for treatment-related adverse events

Physicians interviewed enrolled patients regarding treatment-related adverse events at follow up. Patients were asked for their treatment-related adverse effects type as both of open-ended, and closed-ended questions. Items for closed-ended questions regarding treatment-related side effect types were as follows: (1) Taste disturbance; (2) Nausea/vomiting; (3) Diarrhea/Loose stool/constipation; (4) Abdominal discomfort, dyspepsia; (5) General weakness, myalgia; (6) Dizziness, head ache; and (7) Skin rash.

The degree of treatment-related side effect were classified as 'mild', 'moderate', and 'severe' events according to the degree of tolerance of patients' daily activities as follows: No adverse events; mild (without limitation in daily activities); moderate (partly limited daily activities); and severe (completely limited daily activities). Patients were instructed to visit hospital immediately when any severe adverse events occurred.

Definition for treatment compliance

Treatment compliance was defined according to the status of the consumption of the prescribed drugs through personal interviews at the follow-up visit. The compliance level was investigated through patients' self-reported questionnaire, and consumed/remained medication pill counts. Patients who consumed > 80% of the scheduled prescription were classified as having good compliance.

Statistics

Treatment outcomes (efficacy profiles and safety profiles) were analyzed using an ITT analysis and PP analysis. In the ITT analysis, after excluding patients meeting the exclusion criteria, all of the enrolled study population were included. In PP analyses, patients who were lost to follow-up or those with poor compliance (< 80%) were excluded. Categorical variables were analyzed as percentiles and compared between the TT and CT groups using the chi-square test. Continuous variables were

represented as mean ± SD and were compared between groups using Student's t-test. Statistical significance was set to $P < 0.05$. The Statistical Package for the Social Sciences (SPSS) software (version 22.0; IBM Corp., Armonk, NY, United States) was used for statistical analyses.

RESULTS

Clinical characteristics

Of the 217 patients with a treatment-naive *H. pylori* infection, 110 [men: $n = 55$ (50.00%); mean age: 58.66 ± 13.03] were treated with TT, and 107 with CT [men: $n = 52$ (48.60%); mean age: 56.67 ± 10.88] (Table 1). The rates of endoscopy, smoking, drinking, and comorbidity status were not significantly different between the groups (Table 1).

After evaluating CLR resistance status using DPO-PCR test, of the 101 patients who were initially allocated to the TT group, 29 (26.36%) tested positive for CLR-resistant *H. pylori*, while 81 (73.64%) were classified as CLR-sensitive (Table 1). Among the 29 patients with CLR-resistant *H. pylori*, four patients showed a A2142G point mutation, while the other 25 patients showed a A2143G point mutation (Table 1).

Follow up loss rate and compliance status of TT vs CT group

The follow-up loss (TT *vs* CT; 8.18% *vs* 9.35%, $P = 0.95$) and poor compliance rates (0% *vs* 1.87%, $P = 0.33$) were not significantly different between the groups (Table 1).

Efficacy profiles (ESR) of TT vs CT group

In the ITT protocol, ESR after treatment was not significantly different between the groups (82.73% *vs* 82.24%, $P = 0.95$) (Table 2).

Additionally, in the PP protocol, the TT group showed a lower ESR than the CT group (90.10% *vs* 91.58%, $P = 0.72$), but the difference was not statistically significant (Table 2).

ESR in TT group according to DPO-PCR results

When we determined ESR in the TT group according to the DPO-PCR results, a total of 72 patients showed CLR sensitive results (treated with PAC regimen), with the ESR for those patients being 80.25%, and 87.84% in the ITT and PP analyses, respectively (Table 3). Four patients showed an A2142G point mutation with ESR of (3/4) 75.00% and (3/3) 100% in the ITT and PP analyses, respectively (Table 3). Twenty-five patients with an A2143G point mutation showed an ESR of 92.00% and 95.83% in ITT and PP analyses, respectively (Table 3).

Patient reported treatment-related side effect profiles of TT vs CT group

Treatment-related and patient-reported side effect events were significantly lower in the TT group than in the CT group (22.77% *vs* 50.52%, $P < 0.001$) (Table 4).

The most common side effect was nausea/vomiting [$n = 9$ (10.23%)] in the TT group and taste disturbance [$n = 18$ (18.56%)] in the CT group (Table 4).

DISCUSSION

In this study, we investigated the efficacy (ESR) and safety profiles of TT as a first-line *H. pylori* eradication regimen compared to those of CT in Korea, where the CLR resistance rate is increasing (> 15%). According to our study results, the ESR between the TT and CT groups was not statistically different between the groups. However, the TT group showed a significantly lower treatment-related side effect rate as compared to that of the CT regimen. Considering that lower exposure to antibiotics and appropriate drug use are the best policies for reducing the spread of antibiotic-resistant bacteria[26,27], the TT regimen might be a promising *H. pylori* eradication strategy in an era where increased risk for antibiotic resistance has become a huge medical burden, as is the case in Korea.

Even though the CT regimen for *H. pylori* eradication has shown high ESR in Korea until recently, when compared to that of conventional TT, and even sequential treatment, several concerns arise with this regimen[14,28-34]. First, since the CT regimen includes multiple antibiotic medications for *H. pylori* eradication such as CLR, AMX, and MTZ, there is a possibility of antibiotic overuse[30,31,35]. Given that

Table 1 Baseline characteristics of the study population

	TT group (*n* = 110)	CT group (*n* = 107)	*P* value
Age, mean ± SD (yr)	58.66 ± 13.03	56.67 ± 10.88	0.22
Men, *n* (%)	55 (50.00)	52 (48.60)	0.89
Body mass index (m^2/kg)	24.31 ± 3.23	23.87 ± 2.91	0.35
Smoking, *n* (%)	24 (22.82)	22 (20.59)	0.74
Drinking, *n* (%)	30 (30.00)	28 (26.17)	0.39
Comorbidity			0.81
Hypertension, *n* (%)	26 (23.64)	23 (21.50)	
Diabetes mellitus, *n* (%)	22 (20.00)	25 (23.36)	
Cardiovascular disease, *n* (%)	2 (1.82)	2 (1.86)	
Reasons for eradication			0.37
Peptic ulcer disease	49 (44.54)	43 (40.19)	
Post ESD due to EGC	4 (3.63)	2 (1.87)	
MALToma	0 (0.00)	1 (0.93)	
Chronic atrophic gastritis with intestinal metaplasia	57 (51.82)	61 (57.01)	
Clarithromycin resistance diagnosed by DPO-PCR			
No, *n* (%)	81 (73.64)		
A2142G positive, *n* (%)	4 (3.64)		
A2143G positive, *n* (%)	25 (22.73)		
Follow up loss, *n* (%)	9 (8.18)	10 (9.35)	0.95
Poor compliance, *n* (%)	0 (0.00)	2 (0.02)	0.30

TT: Tailored therapy; CT: Concomitant therapy; DPO-PCR: Dual priming oligonucleotide polymerase chain reaction; ESD: Endoscopic submucosal dissection; EGC: Early gastric cancer; MALToma: Mucosa associated lymphoid tissue lymphoma.

Table 2 *Helicobacter pylori* eradication success rates

Eradication rate	TT group (*n* = 110)	CT group (*n* = 107)	*P* value
Intention-to-treat	91/110 (82.73)	88/107 (82.24)	0.95
Per-protocol	91/101 (90.10)	87/95[1] (91.58)	0.72

[1]Of 107 patients who were initially allocated into concomitant therapy group, 10 were lost to follow-up, and two patients showed poor compliance for taking medication (one patient showed eradication success, and the other eradication failure). Therefore, a total of 95 patients were included in our per-protocol analysis. TT: Tailored therapy; CT: Concomitant therapy.

indiscriminate misuse of antibiotics is related to the emergence of multidrug resistance bacteria, not just limited to *H. pylori*, appropriate use of antibiotics based on drug sensitivity analysis has been emphasized in solving the current multidrug resistance problems of bacteria[27,35]. Even if the CT regimen is effective in *H. pylori* eradication, there is room for antibiotic overuse, which might result in violating antibiotic stewardship, thus physicians should only use a CT regimen with extreme caution[13,27,30,36,37]. Second, because the CT regimen contains multiple antibiotic options, higher incidences of treatment-related side effects, which are related to poor treatment compliance, and ultimately result in eradication failure, have been reported in previous studies[31,32,38,39]. According to our findings, the CT group showed a statistically higher incidence of treatment-related side effects than the TT group.

The paradigm shift from empirically chosen eradication policy to bacteria-specific targeted therapy in *H. pylori* eradication, TT, originated from the rise of precision medicine and ab accumulation of data on resistance mechanisms of *H. pylori* infection [11,15,16,25,37,40-42]. According to a meta-analysis by Venerito *et al*[43] that analyzed

Table 3 Eradication success rate in tailored therapy group according to dual priming oligonucleotide polymerase chain reaction results

Eradication rate	No mutation	A2142G positive	A2143G positive
Number of patients who initially enrolled	81	4	25
Number of patients with eradication failure	9	0	1
Number of patients lost to follow up	7	1	1
Number of patients with poor compliance	0	0	0
Eradication success rate			
Intention-to-treat	65/81 (80.25%)	3/4 (75.00%)	23/25 (92.00%)
Per-protocol	65/74 (87.84%)	3/3 (100%)	23/24 (95.83%)

Table 4 Treatment related adverse events, *n* (%)

	TT group (*n* = 101[1])	CT group (*n* = 97[2])	*P* value
Eradication related Side effects			< 0.001
No	78 (77.23)	48 (49.48)	
Yes	23 (22.77)	49 (50.52)	
Taste disturbance	2 (1.98)	18 (18.56)	
Nausea/vomiting	10 (9.90)	15 (15.46)	
Diarrhea/loose stool/constipation	6 (5.94)	9 (9.28)	
Abdominal discomfort, dyspepsia	3 (2.97)	3 (3.09)	
General weakness, myalgia	1 (0.99)	1 (1.03)	
Dizziness, headache	1 (0.99)	2 (2.06)	
Skin rash	0 (0.00)	1 (1.03)	

[1]Of the 110 patients who were initially allocated into the tailored therapy (TT) group, 9 were not followed up. Therefore, a total of 101 patients were included in the TT group.
[2]Of the 107 patients who were initially allocated into the concomitant therapy (CT) group, 10 were lost to follow up. Therefore, a total of 97 patients were included in the CT group, regardless of compliance status. TT: Tailored therapy; CT: Concomitant therapy.

the effect of antibiotic sensitivity of *H. pylori* on ESR, a conventional triple regimen yielded an ESR of 80%–95% among CLR-sensitive strains, but dropped to 0%–48% among CLR resistant strains[43,44]. Therefore, CLR resistance significantly affected the ESR of the conventional triple regimen[43]. However, MTZ resistance was not significantly associated with the ESR of PBMT even in MTZ-resistant strains as compared to that in MTZ-sensitive strains[43]. Therefore, confirming CLR susceptibility before eradication in order to use a tailored eradication was closely associated with an improved ESR as compared to that of the empirically chosen conventional triple regimen, regardless of the MTZ resistance test results[19-21,41,45-47].

To date, several antibiotic resistance mechanisms of *H. pylori* infection have been proposed[48]. First, antimicrobial genes that are expressed as key targeted structures, such as cell membranes, nucleic acids, DNA gyrases, DNA-dependent RNA polymerases, and redox enzymes, mutate to evade antibiotics, such as quinolone series resistant *H. pylori*. Second, the efflux systems of *H. pylori* change to prohibit the intracellular accumulation of antibiotics. Third, *H. pylori* enzymes that inactivate antibiotic compounds are activated or produced. Among these mechanisms, the CLR resistance phenomenon for *H. pylori* infection mainly originates from the mutation of the cellular target genes, especially associated with protein translation in the V domain of 23S rRNA (A2143G, A2142C, A2142G, A2143C, *etc.*)[48-50]. In our study, we used the DPO-PCR test for CLR resistance detection, which specifically targeted the A2143G and A2142G point mutations. Among the 101 patients in the TT group, 72 of them showed CLR sensitivity, while 29 showed CLR resistance. Even among patients with a negative DPO-PCR test result (*n* = 72), who were classified with wild-type *H. pylori* strains against CLR, and prescribed the conventional triple regimen, the ESRs were

70.83% and 78.46% in the ITT and PP analyses, respectively. This phenomenon might have resulted from mutations in the V domain of 23S rRNA other than A2142G and A2143G point mutations, which were not detected in the DPO-PCR test we used. Considering cost effectiveness, we targeted *H. pylori* strains with the most common site of point mutations resulting in CLR resistance. It is possible that the *H. pylori* strains classified as wild type through the DPO-PCR test were not really wild type. However, among the patients who showed a 2142G or A 2143 G point mutation *via* the DPO-PCR test, the ESR of the PBMT regimen was 100% and 90.48%, respectively, in the PP analysis. Efforts to develop cost-effective and multiple point mutation detection kits for *H. pylori* should be continued in order to improve the ESR of TT[23,24,51].

Even though there is limited data on the efficacy of the TT regimen in *H. pylori* treatment policy, there have been several studies assessing TT efficacy profiles compared to that of the CT regimen[45,52,53]. For example, Ong *et al*[52] conducted a randomized controlled study comparing the treatment outcomes of TT regimen *vs* CT regimen, and reported that in ITT analyses, the TT regimen group showed a higher eradication rate compared the CT regimen group[52]. As for treatment-related side effect events, the TT group also showed fewer events during treatment than the CT group. While this study was conducted in Pusan, Korea, which is far from Incheon, Korea, where the GMC is located, similar treatment outcomes were induced even though the CLR resistance rates differed between the cities.

Furthermore, Lesprit *et al*[27] reported that a tailored *H. pylori* eradication strategy based on the presence of a 23S ribosomal RNA point mutation that causes CAM resistance in patients with *H. pylori* infection is more cost effective than empirical treatment[27]. Kim *et al*[25] also conducted an economic modeling study comparing TR based on DPO-PCR and empirical treatment.

Even efficacy of TT regimen in treating *H. pylori* infection has been evaluated, cost-effectiveness of TT regimen should be evaluated to be widely used in clinical practice in Korea. Tailored regimen needs additional diagnostic procedure of antibiotic resistance test such as DPO-PCR. Even it varies depending on insurance coverages, DPO-PCR costs approximately $55.24 more than rapid urease test in Korea[23]. In this regards, several previous studies investigated medical costs of *H. pylori* tailored eradication strategy as compared to empirical first line eradication strategy (CLR based triple regimen) in Korea[23]. Cho *et al*[23] reported that it is acceptable level of predictive additional costs (only an extra $3.96 *per* eradicated patient) for tailored *H. pylori* eradication strategy with DPO-CPR as compared to CLR based conventional triple therapy[23]. In our study, we compared efficacy and safety level of TT *vs* CT regimen, and should discuss the medical cost effectiveness of TT *vs* CT regimen. However, there has been little data to investigate medical cost of TT strategy as compared to CT regimen. One would say medical cost of TT regimen might be much higher than that of CT regimen, since ESR of empirical CT regimen is generally much higher than empirical CLR based triple regimen. Not just considering each patient's medical cost during *Helicobacter* eradication, but also given that worrisome issues on increased prevalence of drug resistance bacteria in worldwide, tailored approaches in treating *H. pylori* infection should be considered following the major principle of antibiotic use guidelines, antibiotic sensitivity result-based treatment.

Limits of the study

There are several limitations to this study. First, although we prospectively and randomly assigned patients to either the TT or CT group, we retrospectively reviewed the data. Second, since this study was conducted in a tertiary center located in Incheon, Korea, we applied our research results to other regions with caution. In a previous study, researchers investigated the CLR resistance rate for *H. pylori* infection in Incheon, and reported a CLR resistance rate of approximately 30%[54]. Although the aforementioned study was not conducted in our hospital, the GMC, the CLR resistance for *H. pylori* infection is similar to our study. Given that different regions might differ in *H. pylori* infection status and antibiotic resistance status, more multicenter and multinational studies are needed. Third, since we did not culture *H. pylori* for the antibiotic sensitivity test, but instead replaced it with a DPO-PCR test, and are therefore subject to the pit falls of a DPO-PCR. Nevertheless, in a previous study, the DPO-PCR test was validated alongside a culture-based antibiotic resistance test and showed approximately 98% accuracy[20,55].

CONCLUSION

Despite the aforementioned limitations, this study focused on the efficacy and safety profiles of the TT regimen compared to those of the CT regimen in a relatively large dataset in Korea. For the rapidly rising antibiotic resistance rate, the most active countermeasure is to perform an antibiotic resistance test for the strain and actively select the appropriate antibiotic and then decide on the treatment.

In conclusion, according to our study results, the TT regimen showed promising results in terms of efficacy and safety profiles for the first-line regimen of *H. pylori* eradication as compared to those of the CT regimen. Therefore the DPO-based TT regimen might be a successful option for *H. pylori* eradication, especially when physicians are confronted with increased antibiotic resistance rates for *H. pylori* eradication.

ARTICLE HIGHLIGHTS

Research background

Antibiotic resistance to *Helicobacter pylori (H. pylori*) infection has been an emerging issue in the clinical field. Recently, to overcome this problem, an antibiotic sensitivity-based tailored therapy (TT) for *H. pylori* infection has got attention.

Research motivation

However, there is limited data regarding efficacy of TT strategy in treatment of *H. pylori* infection in Korea as compared to that of concomitant therapy (CT) regimen.

Research objectives

To investigate the efficacy and safety profiles of TT for *H. pylori* infection treatment compared to a non-bismuth quadruple therapy, CT.

Research methods

We included treatment naive *H. pylori* infection patients (> 18 years) who visited the Gil Medical Center between March 2016 and October 2020. After randomly assigned to either the TT or CT treatment group in 1 to 1 manner, patient compliance, eradication success rate (ESR), and patient-reported side effects profiles were compared between the two groups. For the TT group, a dual priming oligonucleotide polymerase chain reaction (DPO-PCR) test, which detected A2142G and/or A2143G point mutations, and a clarithromycin (CLR) resistance test were performed. Patients in the CLR-resistant group were treated with a bismuth-containing quadruple combination therapy, while those with sensitive results were treated with the standard triple regimen.

Research results

Of the 217 patients with a treatment naive *H. pylori* infection, 110 patients [mean age: 58.66 ± 13.03, men, n = 55 (50%)] were treated with TT, and 107 patients [mean age: 56.67 ± 10.88, men, n = 52 (48.60%)] were treated with CT. The compliance (TT *vs* CT, 100% *vs* 98.13%, P = 0.30), and follow-up loss rates (8.18% *vs* 9.35%, P = 0.95) were not significantly different between the groups. The ESR after treatment was also not statistically different between the groups (TT *vs* CT, 82.73% *vs* 82.24%, P = 0.95). However, the treatment-related and patient-reported side effects were significantly lower in the TT group than in the CT group (22.77% *vs* 50.52%, P < 0.001).

Research conclusions

The DPO-based TT regimen shows promising results in efficacy and safety profiles as a first-line *Helicobacter* eradication regimen in Korea, especially when physicians are confronted with increased antibiotic resistance rates.

Research perspectives

The DPO-based TT regimen might role as a first-line *Helicobacter* eradication regimen with similar efficacy and safety profiles as compared to CT regimen.

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ORIGINAL ARTICLE

Retrospective Study

Histological differentiation impacts the tumor immune microenvironment in gastric carcinoma: Relation to the immune cycle

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Abstract

BACKGROUND

Various histological types of gastric carcinomas (GCs) differ in terms of their pathogenesis and their preexisting background, both of which could impact the tumor immune microenvironment (TIME). However, the current understanding of the immune contexture of GC is far from complete.

AIM

To clarify the tumor-host immune interplay through histopathological features and the tumor immune cycle concept.

who provided written informed consent for participation in research before their surgery were included in the study.

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.

Conflict-of-interest statement: We have no financial relationships to disclose.

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METHODS

In total, 50 GC cases were examined (15 cases of diffuse GC, 31 patients with intestinal-type GC and 4 cases of mucinous GC). The immunophenotype of GC was assessed and classified as immune desert (ID), immune excluded (IE) or inflamed (Inf) according to CD8+ cell count and spatial pattern. In addition, CD68+ and CD163+ macrophages and programmed death-ligand 1 (PD-L1) expression were estimated.

RESULTS

We found that GCs with different histological differentiation demonstrated distinct immune contexture. Most intestinal-type GCs had inflamed TIMEs rich in both CD8+ cells and macrophages. In contrast, more aggressive diffuse-type GC more often possessed ID characteristics with few CD8+ lymphocytes but abundant CD68+ macrophages, while mucinous GC had an IE-TIME with a prevalence of CD68+ macrophages and CD8+ lymphocytes in the peritumor stroma. PD-L1 expression prevailed mostly in intestinal-type Inf-GC, with numerous CD163+ cells observed. Therefore, GCs of different histological patterns have specific mechanisms of immune escape. While intestinal-type GC was more often related to PD-L1 expression, diffuse and mucinous GCs possessing more aggressive behavior demonstrated low immunogenicity and a lack of tumor antigen recognition or immune cell recruitment into the tumor clusters.

CONCLUSION

These data help to clarify the links between tumor histogenesis and immunogenicity for a better understanding of GC biology and more tailored patient management.

Key Words: Gastric carcinoma; Tumor immune microenvironment; Tumor infiltrating lymphocytes; Tumor associated macrophages

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Core Tip: In this study, we investigated the tumor-host interplay in gastric carcinoma (GC) through the tumor immune cycle concept. Histologically different GCs vary in immunogenicity and differ in tumor-infiltrating lymphocyte and macrophage densities. Intestinal GC demonstrated predominantly inflamed tumor immune microenvironment and frequent programmed death-ligand 1 expression. In contrast, more aggressive diffuse and mucinous GCs possessed low immunogenicity with a lack of cancer antigen recognition and trafficking. These data help to clarify the links between tumor histogenesis and immunogenicity, offering a better understanding of GC biology and the ability to provide more tailored patient management.

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INTRODUCTION

Gastric carcinoma (GC) is one of the leading fatal malignancies worldwide[1]. Currently, GC prognosis and patient management are based on the UICC/American Joint Committee on Cancer (AJCC) tumour-node-metastasis staging system[2]. When assessing tumour prognosis, GC histopathological features are also significant[3]. The Laurén pathohistological classification is the most widely used in clinical practice because it reflects the GC morphology, specifies the tumour biology and behaviour

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and is helpful for treatment decisions[3,4]. However, GC patients with the same TNM stages and similar histopathological features may differ in outcomes[5]. Therefore, the current approach provides incomplete information for patient prognostic stratification and treatment decisions[5]. Recent large-scale studies, including genomic, transcriptomic and proteomic investigations, defined several molecular classifications for GC [6]. The Cancer Genome Atlas Research Network Group[7] suggested a molecular classification that divides GC into four main subtypes, including Epstein-Barr virus (EBV)-positive, microsatellite unstable (MSI), genomically stable and GC with chromosomal instability[8]. At the same time, the Asian Cancer Research Group established another classification with the following types: MSI, microsatellite stable (MSS) with epithelial-mesenchymal transition, MSS/*TP53*+ and MSS/*TP53*−[9]. However, unlike breast cancer, GC molecular classifications have not yet been connected to treatment options[10].

A growing body of evidence has demonstrated the clinical importance of immune cells for tumour prognosis and immunotherapy sensitivity[11]. Tumour-infiltrating immune cells, including neutrophils, macrophages, dendritic cells and different types of T cells, are major contributors to the tumour immune microenvironment (TIME), which modulates tumour development and progression[11]. Previous studies have demonstrated a correlation between the number of tumour-infiltrating lymphocytes (TILs) and the survival of patients with GC[12]. In addition, tumour-associated macrophages (TAMs) are known as alternative regulators of tumour cell proliferation, angiogenesis and tissue remodelling, thereby facilitating metastasis formation in GC [13]. A promising approach is to consider the tumour-host interplay through the mechanisms of tumour immunology and steps of tumour antigen recognition, T-cell activation and tumour cell killing[14].

However, the connections between the histopathological patterns and immune contexture of GC are not well established. This study aims to clarify the interplay among tumour-host immunity, histopathological features and the tumour immune cycle.

MATERIALS AND METHODS

Case selection and patient characteristics

This paper presents a retrospective study that includes 50 cases of primary gastric adenocarcinoma naïve to preoperative chemotherapy or radiotherapy (Table 1). We excluded patients who suffered from other primary malignancies or had a primary tumour of unclear pathological types as well as patients with a known familial or hereditary history of GC. There were 29 males (58%) and 21 females (42%) aged 55.1 ± 3.76 and 45.7 ± 3.59 years, respectively.

The parameters evaluated for each patient included sex, age, tumor location, depth of invasion, grade of differentiation, and tumor staging. Tumor staging was based on the 8th AJCC criteria[15]. Tumor histological type was assessed according to Lauren's classification[3,16]. Among the enrolled cases, there were 15 cases of diffuse GC and 35 patients with intestinal-type GC. The group with intestinal GC included 4 cases of mucinous GC and 31 nonmucinous adenocarcinomas. As mucinous GCs are rare and have a poor prognosis, we assessed them separately.

Tissue processing and immunohistochemistry

The tissues taken after surgery were fixed in 10% neutral buffered formalin and processed. Histological slides were stained by hematoxylin and eosin, as well as by PAS with Alcian blue. For each tumor, the histological subtype and grade were assessed. For immunohistochemistry (IHC), serial sections 4 μm in thickness were used. Tissues were deparaffinized and hydrated. Endogenous peroxidase activity was blocked using 3% methanol in hydrogen peroxide. Next, antigen retrieval in a water bath at 98 °C was performed using Tris EDTA or citrate buffer (pH 6), followed by incubation with primary antibodies. After washing, labeled polymer secondary antibodies (Envision Detection System, Dako) were added to the slides. Peroxidase activity was detected using diaminobenzidine (DAB)–tetrahydrochloride liquid plus Chromogen System (Dako) substrate. The reaction was stopped with distilled water, after which sections were counterstained with hematoxylin and mounted in Richard-Allan Scientific Mounting Medium (Thermo Fisher).

The following antibodies were used for IHC: CD3 (DAKO; polyclonal) for the entire lineage of T lymphocytes; CD8 (DAKO; Clone C8/144B) for cytotoxic T cells as effector cells of cell-mediated antitumor immunity; and CD68 (DAKO, Clone KP1) and CD163

Table 1 Clinical-pathological features of the patients

Characteristics	Intestinal GC	Mucinous GC	Diffuse GC	Total	*P* value
Number	31	4	15	50	
Age	56.3 11.2	51.3 7.37	46.0 18.3	52.8 13.7	0.174
Sex, *n* (%)					0.738
Males	18 (58.1)	3 (75)	8 (53.5)	29 (58)	
Females	13 (41.9)	1 (25)	7 (46.7)	21 (42)	
Stage, *n* (%)					0.33
Stage 2	2 (6.5)	0	0	2 (4)	
Stage 3	13 (41.9)	1 (25)	6 (40)	20 (40)	
Stage 4	16 (51.6)	3 (75)	9 (60)	28 (56)	
Grade, *n* (%)		-	-		
G1	5 (16.1)				
G2	11 (35.5)				
G3	15 (48.4)				
MMR status					
MSI	2	-	-	2	
MSS	29	4	15	48	

GC: Gastric carcinoma; MMR: Mismatch repair; MSI: Microsatellite unstable; MSS: Microsatellite stable.

(Cell Marque, Clone MRQ-26) to visualize TAMs of M1 and M2 phenotypes. In addition, mismatch repair (MMR) deficiency was assessed using antibodies against MLH1 (Clone ES05, DAKO), MSH2 (Clone FE11, DAKO), PMS2 (Clone EP51, DAKO), and MSH6 (Clone EP49, DAKO). Tumors with a lack of MMR enzyme expression in the tumor cell nuclei were defined as MMR deficient or microsatellite unstable (MSI). Staining of stromal and immune cell nuclei was considered a positive control. Additionally, programmed death-ligand 1 (PD-L1) [PDL Cell Signaling, clone E1L3N(R)] expression was detected immunohistochemically. Human tonsil tissue was used as a positive control.

Methodology of tumor-host immunity assessment

To interpret the tumour-host interplay and mechanisms of immune escape, we assessed the TIME according to the immune cycle concept[17]. The following types of TIME were considered: Immune desert, immune-excluded and inflamed. The immune desert (ID) type demonstrates a lack of pre-existing immunity and a few T-cells inside and around the tumour. The immune-excluded (IE) type shows prominent peritumour infiltration but sparse intratumour T lymphocytes. The inflamed (Inf) TIME possesses high lymphocyte infiltration, thus reflecting the activation of antitumour T cells with improper functioning.

When assessing different immune cells, we examined their number and spatial distribution. The primary histopathological assessment was performed microscopically by two independent pathologists. In addition, digital images of the sections were captured using a digital slide scanner (3DHISTECH, Budapest, Hungary) and assessed blindly. The density of CD8+ cells was counted inside the tumour clusters (TCs) and in the peritumour stroma (TS) in 10 visual fields corresponding to "hot spots", with further quantification *per* 1 mm^2.

The number of immunopositive cells was assessed as both continuous and dichotomized variables using cut-off values (84 cells *per* mm^2 as a median) to separate low-density from high-density results. The ID type of TIME was defined in cases with low CD8+ infiltration in both the TC and TS. The cases of the IE immunophenotype included tumours with a low CD8+ quantity in the TC compartment and high CD8+ infiltration in the TS compartment. GC with high CD8+ cell infiltration in the TC and TS was classified as belonging to the Inf immunophenotype. The same approach was used to evaluate the TAM distribution by counting CD68+ and CD163+ cells in the TC

and TS. Immune cells in vessels, submucosal lymphatic areas, and necrosis/necrosis adjacent areas were not counted in this study. The expression of PD-L1 was evaluated using the combined positive score (CPS) according to the percentage of stained tumour or immune (lymphocytes and macrophages) cells, with a positive score defined at CPS ≥ 1%[16].

Statistical analysis

The descriptive statistics included count and frequencies for categorical variables and the mean ± SEM with 95% confidence interval (CI) for continuous variables. Normally distributed continuous variables were analysed with unpaired sample *t*-tests. Nonnormally distributed continuous variables were assessed with nonparametric Mann-Whitney *U*-tests. Chi-square or Fisher's exact test was used to compare categorical variables, whereas one-way analysis of variance was used to compare the continuous variables. Correlations were assessed using the Pearson correlation coefficient. Statistical assessment of the data was carried out using the MedCalc software package. Statistical significance was accepted at $P < 0.05$.

RESULTS

We did not find sex-related differences in the frequency of various histological types of GCs. However, significant differences were observed in the age of patients with diffuse and intestinal GC types, which were 41.1 ± 6.51 (95%CI: 25.2-57.1) and 53.3 ± 3.07 (46.8-59.8) years old, respectively. However, age differences were not observed between patients with mucinous and nonmucinous intestinal GC.

TIL and TAM densities varied in GC of different histological types

The assessment of GC immune profiles revealed that CD3+ and CD8+ lymphocyte counts varied in GCs with different histological types. However, CD8+ cell density did not correlate with the tumour grade ($P = 0.669$) or stage ($P = 0.560$). GCs of various histological differentiation types differed in the density of TILs (Figure 1 and Table 2). The number of CD3+ and CD8+ lymphocytes in intestinal-type GC was significantly higher than that in diffuse and mucinous GCs ($P < 0.001$). Importantly, mucinous GCs demonstrated prominent heterogeneity of immune cell infiltration, with few cells within tumour clusters and a higher density around them.

Compared with the common concept that TAMs correspond to M2 macrophages, in GCs, we found that M1 macrophages prevailed over the M2 type. Significant differences were not observed in CD68+ macrophage infiltration in the peritumour stroma with regard to the histological subtypes of GC ($P = 0.471$). However, the number of CD68+ cells within the tumour cluster was higher in intestinal and diffuse GC than in mucinous GC ($P < 0.001$). Moreover, M1 macrophages were more numerous than CD8+ cells in both intra- and peritumour areas of most observed tumours, and they were even more conspicuous in diffuse and mucinous GC. In contrast, CD163+ cells were less frequent and demonstrated specific associations with distinct histological types. M2 macrophages were few in mucinous and diffuse tumours; however, CD163+ cells were much more numerous in intestinal-type GCs ($P = 0.032$). Notably, the M2 macrophage number was comparable to the CD8+ cell count in all GC types. Notably, GCs with a poor prognosis (mucinous and diffuse type) demonstrated a proinflammatory macrophage phenotype in which the number of CD68+ cells was several times higher than that of M2-type macrophages ($P < 0.001$).

Therefore, CD68+ macrophages were the predominant type of immune cells in GCs of all histological types. The density of TILs demonstrated a relationship with the distinct immune contexture. Intestinal-type GCs were rich in CD68+, CD8+ and CD163+ cells. Diffuse GCs were heavily infiltrated by CD68+ cells but low in CD8+- and CD163+ cells. In mucinous GCs, CD68+ cells were the most abundant and prevailed over CD8+ and CD163+ cells, although the immune cell number was significantly lower within the TC than in the peritumor stroma.

Immunophenotyping GC of different histological types

The histological types of GCs correlated with distinct immunophenotypes ($P < 0.001$) assessed in line with the immune cycle concept (Table 3). There were 10 cases of ID (20%), 13 tumors with IE (26%) and 27 cases with Inf-TIME (54%). Notably, the majority of intestinal-type GCs had an inflammatory TIME (25 of 31 patients; 80.7%). The rest of the patients with intestinal-type GCs showed ID (1 of 31; 3.2%) or IE TIME (5 of 31; 16.1%). More than half of diffuse-type GCs had ID TIME (8 of 15 patients;

Table 2 Immune cells number in gastric carcinoma of different histological types and tumor immune microenvironment

Characteristics	CD8+ cells		CD68+ cells		CD163+ cells	
	TC	TS	TC	TS	TC	TS
Histological type						
Intestinal	214 ± 44.9	202 ± 14.7	303 ± 21.7	244 ± 12.4	173 ± 17.3	170 ± 9.67
	124-305	173-232	259-347	219-269	138-208	151-190
Diffuse	49.5 ± 6.63	66.0 ± 6.03	339 ± 23.6	225 ± 23.8	51.7 ± 8.60	55.5 ± 8.94
	36.0-63.1	43.6-98.3	291-388	176-274	34.1-69.4	37.2-73.9
Mucinous	7.53 ± 2.50	52.5 ± 37.5	101 ± 59.0	219 ± 16.2	12.5 ± 11.8	62.5 ± 12.5
	2.26-34.3	23.9-82.9	64-125	126-264	6.32-78.2	36-121
	$P = 0.045$	$P = 0.059$	$P = 0.071$	$P = 0.471$	$P = 0.032$	$P = 0.011$
TIME type						
ID	43.7 ± 4.05	43.2 ± 5.93	367.5 ± 36.9	213.1 ± 35.9	72.5 ± 18.3	48.7 ± 10.5
	35.1-52.3	30.6-55.7	288-446	135-290	33.2-111	26.2-71.2
IE	19.4 ± 3.43	112 ± 15.2	157.3 ± 29.6	266.6 ± 25.8	55.5 ± 13.6	119.5 ± 16.3
	12.2-26.7	80.1-144	94.5-220	211-321	26.5-84.4	84.9-154.1
Inflamed	229 ± 44.9	190 ± 16.5	352.8 ± 15.6	232.6 ± 13.7	165.6 ± 18.2	151.3 ± 12.1
	138-319	156-223	321-384	204-260	128-202	126-175
	$P = 0.042$	$P = 0.004$	$P = 0.674$	$P = 0.060$	$P = 0.024$	$P = 0.011$
Status PD-L1 expression						
Positive	341 ± 72.3	204 ± 52.7	430 ± 27.7	200 ± 8.19	231 ± 54.3	135 ± 29.5
	66.3-488	58.4-350	341-518	174-226	58.4-404	41.3-229
Negative	49.0 ± 13.1	116 ± 20.7	261 ± 41.4	279 ± 37.9	96.3 ± 28.0	131 ± 28.2
	20.1-77.9	72.0-159	169-352	195-362	34.6-157	69.5-193
	$P = 0.010$	$P = 0.075$	$P = 0.252$	$P = 0.715$	$P = 0.032$	$P = 0.260$

TC: Tumor clusters; TS: Peritumor stroma; TIME: Tumor immune microenvironment; PD-L1: Programmed death-ligand 1; ID: Immune desert; IE: Immune-excluded.

53.3%). The rest were IE (5 of 15; 33.3%) or had an inflammatory TIME (2 of 15; 13.3%). Most mucinous GCs (3 of 4; 75%) were IE TIME, and one case represented the ID immunophenotype. We did not find any statistically significant relationship between TIME and tumor grade ($P = 0.523$) or stage ($P = 0.756$). There were two intestinal-type GCs with MMR deficiency. Both cases were of inflamed-TIME and did not differ significantly in terms of immune cell patterns from other intestinal-type GCs. Therefore, GCs of different histological types illustrated the prevalence of distinct immunophenotypes. Inflamed TIME was more common for intestinal GCs, IE TIME prevailed in mucinous adenocarcinomas, and ID TIME was more typical for diffuse-type GC.

Tumor-infiltrating macrophages in GCs with different TIMEs

Naturally, ID-type GCs demonstrated a low number of CD8+ cells with a similar rate of M2 macrophages. At the same time, the count of CD68+ cells was several times higher than the lymphocyte density ($P < 0.001$). In contrast, GCs with the IE immunophenotype had a centrifugal pattern of immune cell distribution with a twofold lower CD68+ cell count inside tumour clusters compared to peritumour areas ($P < 0.05$). Although CD68+ macrophages were the most abundant immune cells in both the intratumour and peritumour compartments of IE-GCs, their number was twice as low as that in carcinomas with ID and inflammatory TIME ($P < 0.001$). Notably, GCs of the inflamed TIME demonstrated the highest density of CD8+ lymphocytes and CD163+ macrophages in both the tumour cluster and peritumour stroma compared to GCs with other immunophenotypes (Table 2).

Table 3 Relationship between immunophenotype and histological pattern of gastric carcinoma

Characteristics	ID TIME	IE TIME	Inf TIME	Total number
Histological types of GC, *n* (%)				
Intestinal	1 (3.2)	5 (16.1)	25 (80.7)	31 (62)
Diffuse	8 (53.3)	5 (33.3)	2 (13.3)	15 (30)
Mucinous	1 (25)	3 (75)	0	4 (8)
				$P < 0.001$
PD-L1 expression status, *n* (%)				
PD-L1 positive GCs	1 (5.6)	0	8 (94.4)	9 (18)
PD-L1 negative GCs	9 (21.9)	13 (31.8)	19 (46.3)	41 (82)
Total number	10	13	27	50
				$P < 0.001$

TIME: Tumor immune microenvironment; PD-L1: Programmed death-ligand 1; GC: Gastric carcinoma; ID: Immune desert; IE: Immune-excluded.

We did not find any tight association between the number of CD68+ cells and the T lymphocyte infiltration pattern. GCs of ID and inflamed TIME demonstrated the highest number of intratumor CD68+ cells with a smaller amount in peritumor stroma (Figure 2). In contrast, the CD163+ cell number was more tightly associated with GC morphology and TIL patterns. M2 macrophages were the most numerous in intestinal-type GC with an inflamed TIME (Table 2). In addition, their amount was associated with PD-L1 expression.

PD-L1 expression in GCs with different immunophenotypes

To clarify the potential mechanisms of GC immune evasion and its relationship with the TIME, we evaluated PD-L1 expression. There were 9 PD-L1-positive GCs of 50 observed cases (18%), and 8 of them were intestinal-type GCs (8 of 9). We did not find a significant relationship between PD-L1 expression and variables such as age, sex, pathological stage and GC histological type and grade. However, PD-L1 expression was related to TIME type ($P = 0.05$). Most cases of PD-L1 positivity were associated with an inflamed TIME (Table 3 and Figure 3). For tumour-infiltrating immune cells, PD-L1 positivity correlated with high CD8+ cell counts in TCs ($P = 0.005$) and CD163+ macrophages ($P = 0.032$) but not CD68+ density. Additionally, there were two intestinal-type GCs with Inf-TIME demonstrating MMR deficiency, and one of those tumours was PD-L1 positive. Thus, PD-L1 expression was found mostly in GC with an inflamed TIME, intestinal histology and a high density of CD8+ and CD163+ cells. Preexisting chronic inflammation and M2 macrophage polarization may be related to the activation of immune escape mechanisms in GC with an inflamed TIME.

DISCUSSION

The results of the study showed a close relationship between GC histological differentiation and immunophenotype. GCs of distinct histological types differed in terms of the density and spatial patterning of lymphocytes and macrophages, thus reflecting the divergence of the TIME among GCs. Several years ago, three broad classes of the TIME (namely, ID, IE, and Inflamed), representing alterations in consequent steps of the tumour immune cycle, were proposed[17]. According to this concept, ID TIME is characterized by a low number of CD8+ cells caused by poor tumour immunogenicity. This type was typical for diffuse carcinoma and could be due to low mutational load or a lack of antigen release. Alternatively, dendritic cell insufficiency or disrupted costimulatory interactions can diminish antigen presentation and T cell activation[12]. The immune-excluded phenotype, which is more common for mucinous GCs, was associated with the abundance of CD8+ cells in the peritumour stroma; however, T-cytotoxic cells were not able to reach tumour cells. Such a failure can be due to altered mechanisms of T lymphocyte recruitment inside the tumour, which can be caused by the lack of chemokines or low levels of chemokine receptors[18,19]. In contrast, the

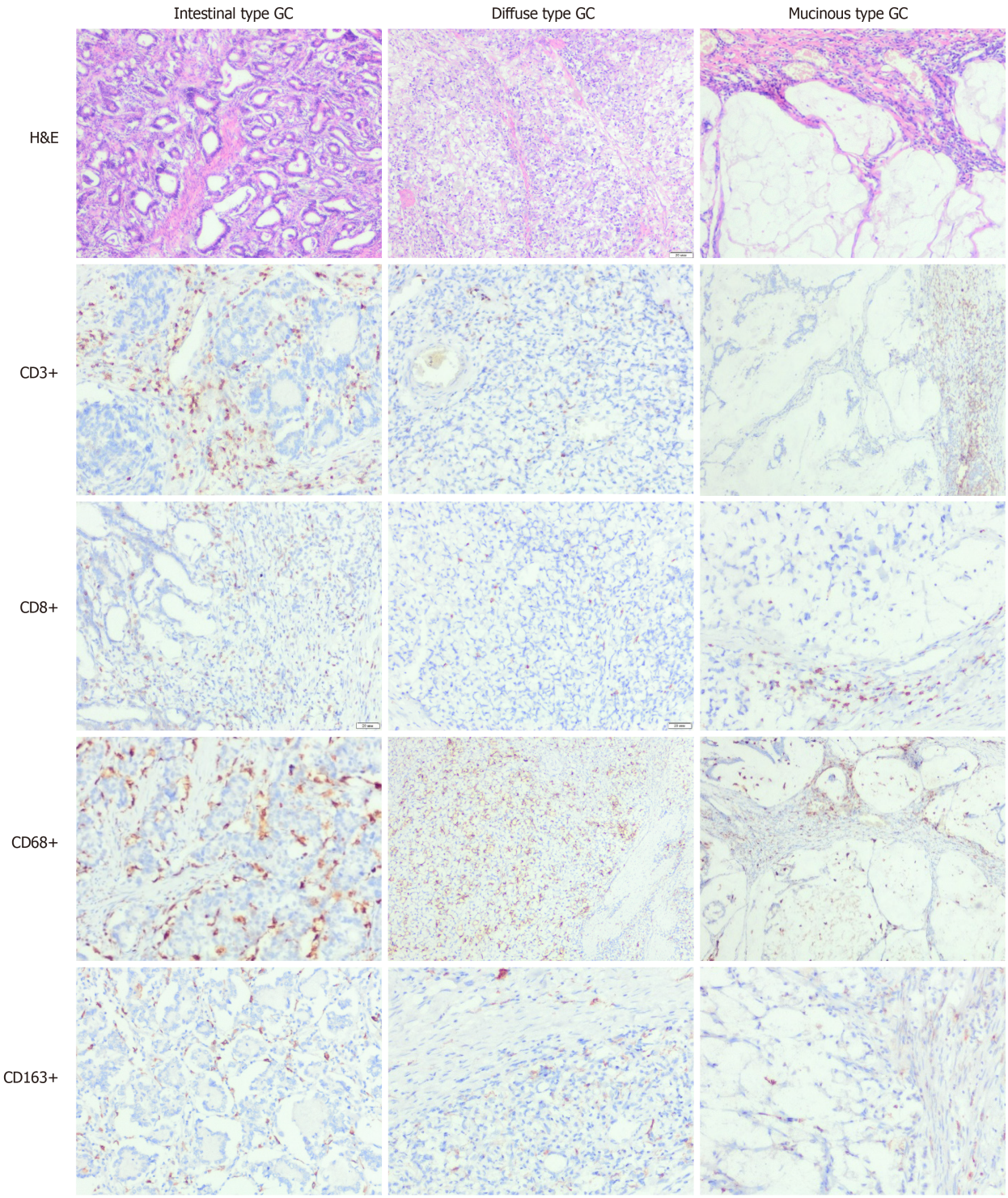


Figure 1 Density of immune cells in gastric carcinoma of different histological types. H&E and immunohistochemistry (× 100). Figures demonstrate differences in infiltration of various histological type gastric carcinoma by immune cells, including entire population of T-lymphocytes (CD3), T-cytotoxic cells (CD8), M1 and M2 macrophages (CD68 and CD163 respectively). GC: Gastric carcinoma.

inflammatory TIME predominated in intestinal-type GCs and was rich in lymphocytes infiltrating tumour clusters. Although these tumours demonstrated an "immune hot" phenotype, it seems that the functioning of TILs was improper[20,21]. In addition, the elevated expression of immunosuppressive molecules, such as VEGF and TGF-β, can make antitumour immunity ineffective[14,21]. Therefore, cancer cells of various differentiation types use specific strategies of immune escape and can block either the early steps of the antitumour response or the final mechanisms of cancer cell killing[22,23].

Why mucinous and diffuse GCs exhibit more invasive behavior remains unclear. It might be related to low cell differentiation, which is associated with a higher rate of proliferation, cell motility and cancer dispersion[24]. However, there is an alternative

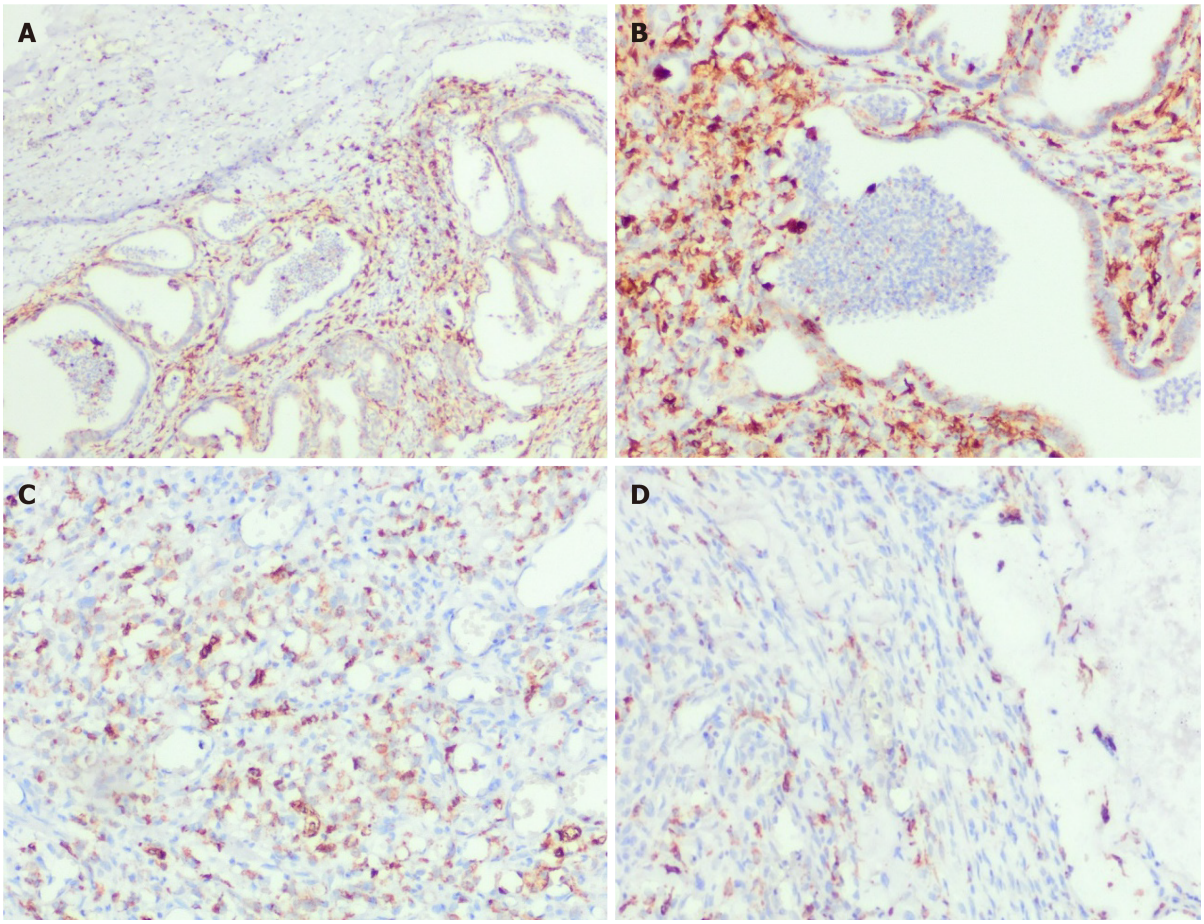


Figure 2 Density and spatial distribution of macrophages in gastric carcinoma of different histological types. Immunohistochemistry for CD68. A and B: Intestinal type gastric carcinoma (GC) of inflamed tumor immune microenvironment (TIME) (A) with high count of CD68+ cells forming dense meshwork (B) inside the tumor. (A: × 100, B: × 200); C: High number of intratumor CD68+ cells of diffuse type GC of ID TIME, × 200; D: Prevalence of CD68+ cells in peritumor stroma of mucinous GC of IE TIME, × 200.

explanation based on immune-mediated mechanisms. Papadopoulos *et al*[25] stated that mucin interferes with the inflammatory response and immunological recognition of tumor cells[25]. Our study supports this hypothesis, demonstrating the disruption of either antigen-presenting cell functioning or CD8+ cell trafficking into the tumor in diffuse and mucinous GCs. As there are no molecular targets identified for diffuse and mucinous GCs, it seems that elucidation of their immunosuppressive potential is a promising issue for further investigation.

In contrast, intestinal-type GC demonstrated a predominantly inflamed TIME that looks natural because it arose from preexisting immunological impairments[26,27]. The chronic inflammatory response is believed to be required for a sequence of epithelial transformations called the Correa cascade, which includes multifocal atrophic gastritis, intestinal metaplasia, dysplasia, and cancer[28-31]. Despite the high density of TILs, the inflamed TIME is often associated with the arrest of antitumour immunity[17,32], which can be provoked by various factors, such as the lack of a major histocompatibility complex in tumour cells, the impact of immunosuppressive immune cells (T-regulatory lymphocytes and myeloid-derived suppressor cells), or the expression of immune checkpoints[33]. Previously, an association between PD-L1 expression and MSI and EBV molecular subtypes was noticed[18]. In this study, we found a link between PD-L1 expression and inflamed TIME predominating in intestinal-type GCs. It is also worth noting that most PD-L1-positive cases were related to the high density of M2 macrophages, which could reflect the role of M2 macrophages in immune escape mechanisms.

Finally, this study revealed the prevalence of CD68+ macrophages in the TIME of GCs. Macrophages are thought to be key players in the innate immune response that could modulate chronic inflammatory responses and carcinogenesis. After monocyte recruitment and transformation, macrophages adjust to particular environmental conditions and adopt either proinflammatory (M1) or anti-inflammatory (M2) phenotypes[34]. It is widely accepted that cancer-associated macrophages are

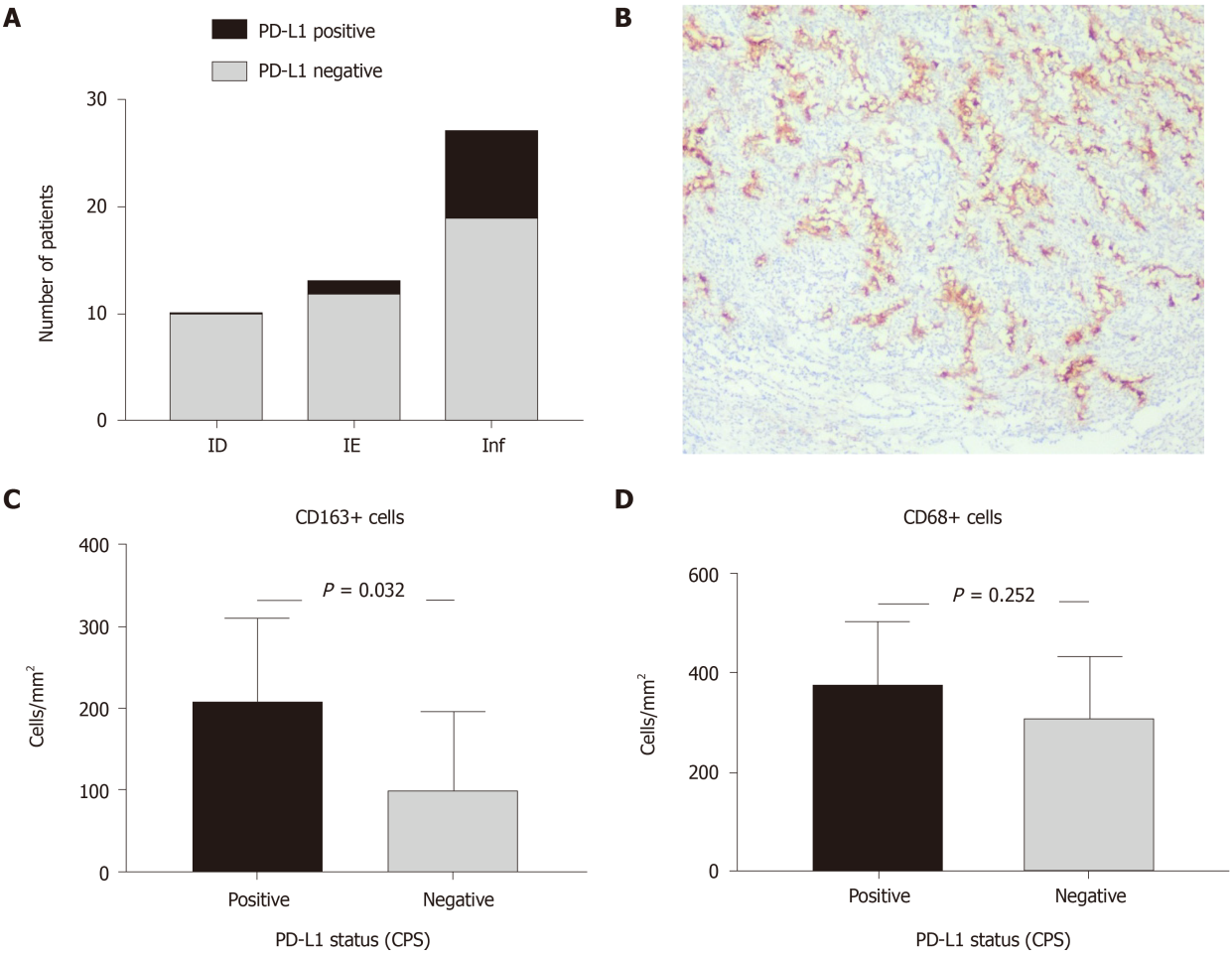


Figure 3 Relationship between programmed death-ligand 1 expression, gastric carcinoma immunophenotype and number of M1 and M2 macrophages. A: Frequency of programmed death-ligand 1 (PD-L1) expression in gastric carcinoma (GC) of different tumor immune microenvironment (TIME); B: PD-L1 expression in tumor cells of Inflamed TIME GC, immunohistochemistry for PD-L1, × 50; C and D: number of M1 (C) and M2 (D)-macrophages in GC regarding PD-L1 expression. PD-L1: Programmed death-ligand 1; CPS: Combined positive score.

represented by the M2 type, which contributes to the suppression of adaptive immunity, tissue remodelling, angiogenesis and tumour growth[33]. In contrast, in this study, we found that GCs were associated with the prevalence of M1 macrophages possessing proinflammatory features. CD68+ cells were the predominant cell type of the TIME and were much more numerous than lymphocytes, particularly in diffuse GCs. On the one hand, this finding demonstrates the role of innate *vs* adaptive immune cell balance in carcinomas of various aggressiveness levels. On the other hand, it conflicts with the concept of the high proinflammatory and antitumour activity of M1 macrophages because a high number of these macrophages can improve patient outcomes. However, in diffuse GC, the loss of cadherin expression in cancer cells combined with M1 macrophage activity (reactive oxygen species, causing additional DNA damage and mutagenesis, and matrix-degrading enzymes) could build an effective strategy against adverse biological cancer behaviour. This hypothesis motivates a greater understanding of the role of M1 macrophages in gastric cancer prognostication.

Our study is limited by its retrospective design and descriptive nature as well as the small number of cases for each tumour subtype, and these limitations preclude multivariate analyses of the immunologic variables and clinical outcomes. In addition, the cloning of antibodies used in the study to evaluate PD-L1 expression is not currently used in clinical practice to select gastric cancer patients for immunotherapy.

CONCLUSION

In this study, we found a relationship between the histological and immunological

features of GC. Various histological types of GC demonstrate distinct immunophenotypes that could be related to different pathways defining tumor immunogenicity and mechanisms of immune evasion. Diffuse and mucinous GCs have low immunogenicity, which is associated with a lack of antitumor immunity activation or T cell recruitment but dense infiltration by CD68+ macrophages. In contrast, intestinal-type GCs exhibit a predominantly inflamed TIME associated with M2 macrophage polarization and PD-L1 expression.

ARTICLE HIGHLIGHTS

Research background

Various histological types of gastric carcinomas (GCs) differ in terms of their pathogenesis and preexisting background, both of which could impact the tumour immune microenvironment (TIME). However, the current understanding of the immune contexture of GC is far from complete.

Research motivation

The data can help to clarify the links between tumor histogenesis and immunogenicity, offering a better understanding of GC biology and the ability to provide more tailored patient management.

Research objectives

This retrospective study aimed to clarify the tumor-host immune interplay through histopathological features and the tumor immune cycle concept.

Research methods

Of 50 GC cases were examined (15 cases of diffuse GC, 31 patients with intestinal-type GC and 4 cases of mucinous GC). The immunophenotype of GC was assessed and classified as immune desert (ID), immune excluded (IE) or inflamed (Inf) according to CD8+ cell count and spatial pattern. In addition, CD68+ and CD163+ macrophages and programmed death-ligand 1 (PD-L1) expression were estimated.

Research results

GCs with different histological differentiation demonstrated distinct immune contexture. Most intestinal-type GCs had inflamed TIMEs rich in both CD8+ cells and macrophages. In contrast, more aggressive diffuse-type GC more often possessed ID characteristics with few CD8+ lymphocytes but abundant CD68+ macrophages, while mucinous GC had an IE-TIME with a prevalence of CD68+ macrophages and CD8+ lymphocytes in the peritumor stroma. PD-L1 expression prevailed mostly in intestinal-type Inf-GC, with numerous CD163+ cells observed.

Research conclusions

GCs of different histological patterns have specific mechanisms of immune escape. While intestinal-type GC was more often related to PD-L1 expression, diffuse and mucinous GCs possessing more aggressive behavior demonstrated low immunogenicity and a lack of tumor antigen recognition or immune cell recruitment into the tumor clusters.

Research perspectives

Assessment of tumor immune microenvironment in complex with the molecular landscape will provide a deeper understanding of the links between tumor histogenesis and immunogenicity for a better understanding of GC biology and more tailored patient management.

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ORIGINAL ARTICLE

Observational Study

Prediction of the severity of colorectal lesion by fecal hemoglobin concentration observed during previous test in the French screening program

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Author contributions: The study was conceived and designed by Balamou C and Koïvogui A; the data was acquired and collated by Balamou C, Clerc A, Piccotti C, Deloraine A, and Exbrayat C; the data were analyzed by Balamou C, Koïvogui A, and Rodrigue CM; the study was drafted and revised critically by all authors; All authors gave final approval of the version to be published and have contributed to the study.

Institutional review board statement: This study is co-signed by the heads of the structures involved, as such, no further Institutional Review Board was required.

Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous data that was obtained

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Abstract

BACKGROUND

The rate of positive tests using fecal immunochemical test (FIT) does not decrease with subsequent campaigns, but the positive predictive value of advanced neoplasia significantly decreases in subsequent campaign after a first negative test. A relationship between the fecal hemoglobin concentration (Fhb) and the opportunity to detect a colorectal cancer in subsequent campaign has been shown.

AIM

To predict the severity of colorectal lesions based on Fhb measured during previous colorectal cancer screening campaign.

METHODS

This etiological study included 293750 patients aged 50-74, living in Auvergne-Rhône-Alpes (France). These patients completed at least two FIT [$test_{(-1)}$ and $test_{(0)}$] between June 2015 and December 2019. Delay between $test_{(-1)}$ and $test_{(0)}$ was > 1

after each patient agreed to participate in screening campaigns.

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year and test$_{(-1)}$ result was negative (< 150 ngHb/mL). The severity of colorectal lesions diagnosed at test$_{(0)}$ was described according to Fhb measured at test$_{(-1)}$ [Fhb$_{(-1)}$]. The relationship between the severity classified in seven ordinal categories and the predictive factors was analyzed in an ordered multivariate polytomous regression model.

RESULTS

The test$_{(0)}$ positive rate was 4.0%, and the colonoscopy completion rate was 97.1% in 11594 patients who showed a positive test$_{(0)}$. The colonoscopy detection rate was 77.7% in those 11254 patients who underwent a colonoscopy. A total of 8748 colorectal lesions were detected (including 2182 low-risk-polyps, 2400 high-risk-polyp, and 502 colorectal cancer). The colonoscopy detection rate varied significantly with Fhb$_{(-1)}$ [0 ngHb/mL: 75.6%, (0-50 ngHb/mL): 77.3%, (50-100 ngHb/mL): 88.7%, (100-150 ngHb/mL): 90.3%; P = 0.001]. People with a Fhb$_{(-1)}$ within (100-150 ngHb/mL) (P = 0.001) were 2.6 (2.2; 3.0) times more likely to have a high severity level compared to those having a Fhb$_{(-1)}$ value of zero. This risk was reduced by 20% in patients aged 55-59 compared to those aged < 55 [adjusted odds ratio: 0.8 (0.6; 1.0)].

CONCLUSION

The study showed that higher Fhb$_{(-1)}$ is correlated to an increased risk of severity of colorectal lesions. This risk of severity increased among first-time participants (age < 55) and the elderly (≥ 70). To avoid the loss of chance in these age groups, the FIT positivity threshold should be reduced to 100 ngHb/mL. The other alternative would be to reduce the time between the two tests in these age groups from the current 2 years to 1 year.

Key Words: Colorectal cancer screening; Fecal immunochemical test; Fecal hemoglobin concentration; Colorectal lesion severity

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Core Tip: The study showed that the severity of the colonic lesions increases with a high concentration of fecal hemoglobin measured in previous test. The elderly (≥ 70 years) had a high proportion of positive colonoscopy when the fecal hemoglobin concentration measured in previous campaign was between 100 and 150 ngHb/mL. Younger patients (age < 54) were likely to have a high-severity neoplasia. Given these results, the recommendation to reduce the FIT positivity threshold to 100 ngHb/mL for first-time participants and the elderly (aged ≥ 70) should attract the attention of the decision-making authority.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of cancer-related death[1]. To address these major public health issues, several countries have launched nationwide CRC screening program (CRCSP) in order to reduce the incidence and mortality of CRC.

In 2015, in France, the fecal immunochemical test (FIT) OC-Sensor® was introduced as part of the CRCSP based on its performance to detect advanced adenoma and CRC [2-5]. The positivity threshold has been set at 150 ngHb/mL (30 μgHb/g) of stool. Anyone who tested negative was re-invited for testing 2 years later. Anyone who tested positive was advised to complete a colonoscopy. Several studies showed a

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reduction in the CRC incidence several years after FIT introduction in the CRCSP[6,7].

Some colonic polyps such as adenomatous and serrated polyps exhibit a malignant potential, while others do not (hyperplastic, post-inflammatory, hamartomatous)[8]. Surveillance of polyp characteristic over time should lead to a significant increase in the clinical performance of the detection of polyps with high malignant potential[9]. The variability in growth behavior supports the hypothesis that some polyps continue to grow, other are remaining stable, whereas some polyps regress over time. Some authors have reported that advanced adenoma may have the potential to grow faster than non-advanced adenoma, while most small polyps remain stable or are regress over time[10,11].

It was shown that the rate of positive test results with FIT does not decrease during the subsequent campaigns, but the positive predictive value for advanced neoplasia, especially for CRC, significantly decreased in participants during the subsequent campaign after a first negative test[12,13]. A relationship between the fecal hemoglobin concentration (Fhb) and the risk to detect a CRC or an advanced adenoma has also been shown[14-21], but the severity risk of the colorectal lesions according to the earlier Fhb was poorly described.

Looking for less invasive methods for monitoring patients at moderate risk for CRC, this study aims to predict the severity of colorectal lesions based on the Fhb measured during a previous colorectal cancer screening test.

MATERIALS AND METHODS

Study design and population

This etiological study included 293750 patients living in one of the six French departments (Ain, Ardèche, Drôme, Isère, Savoie, & Haute-Savoie) of the Auvergne-Rhône-Alpes region. These patients were aged between 50 and 74 and had completed at least two FIT screening tests between June 1, 2015 and December 31, 2019. The delay between the last test [$test_{(0)}$] and the penultimate test [$test_{(-1)}$] was > 1 year, with a negative $test_{(-1)}$ result (< 150 ngHb/mL). The diagnosis of a colorectal lesion as well as its severity were described according to the Fhb measured at $test_{(-1)}$ [$Fhb_{(-1)}$].

All study data were extracted on the same date (November 30, 2020) from departmental databases. These databases were regularly enriched by socio-demographic data, diagnosis (colonoscopy, histopathology), and follow-up data provided by partners (Health Insurance Plans, Medical Information Services, Gastroenterologists, Surgeons & GPs).

Population and design

In each selected department, the CRCSP campaigns were organized according to the CRCSP national specifications[22,23]. As a reminder, this nationwide screening program started in 2009, and the CRCSP target was every 2 years in an eligible population, *i.e.* asymptomatic patients aged 50 to 74, with no risk factors other than their age (according to the CRCSP national specification). This program was based on the guaiac fecal occult blood test (gFOBT) or hemoccult© II, which has been replaced by the FIT in 2015. The CRC screening test was a two-step method: The first step consisted in the completion of a FIT, the second-step consisted in the completion of a colonoscopy in case of positive FIT. The positivity threshold of the test was set at 150 ngHb/mL of stool. In case of normal colonoscopy, the patient received an invitation to the CRCSP after 5 years. In case of positive colonoscopy (high risk polyps or cancer) the patient was excluded from the nationwide program.

Operational definition of variables and descriptive analysis

As a study result criterion, $test_{(0)}$ was positive at the threshold of 150 ngHb/mL. The value of the Fhb measured during this $test_{(0)}$ [$Fhb_{(0)}$] was treated as a discrete variable (0 ngHb/mL, 0-50 ngHb/mL, 50–100 ngHb/mL, 100-150 ngHb/mL, 150-300 ngHb/mL, > 300 ngHb/mL). The colonoscopy completion rate was defined by the proportion of patients who had a colonoscopy (complete or incomplete) among those with a positive $test_{(0)}$ result. Colonoscopy was considered positive when a colorectal neoplasia was diagnosed by the gastroenterologist or by a cytopathological examination of the specimens. In the CRCSP, no specific training other than specialized training as an endoscopic physician was required for the practice of colonoscopy by gastroenterologists. The colonoscopy detection rate was defined by the proportion of positive colonoscopies among those performed (complete or incomplete) after a positive test. In the event of a positive result, the diagnostic course was analyzed in terms of types of

diagnosed lesions including: Low risk polyps (LRP), high risk polyps (HRP), unspecified-polyp (UP), and CRC. HRP included: Adenomas ≥ 10 mm (except hyperplastic polyps), serrated adenomas, adenomas with high grade dysplasia, and villous or tubulo-villous adenomas. For these colorectal tumors detected, five localizations were described during colonoscopy: Cecum, right colon (ascending colon and right angle), transverse colon, left colon (left angle and descending colon), and rectosigmoid (rectum and sigmoid colon). The diagnoses associated with CRC and polyps or adenoma were those related to C18–C20 and D12 of the 10th version of the World Health Organization International Classification of Diseases[24]. CRC lesions were described by stage of severity, using the tumor, node, and metastasis (TNM) classification based on tumor size, lymph node involvement, and the possible presence of metastases[25]: CRC Stage-0 ($pTisN_0M_0$), Stage-I ($pT_{1-2}N_0M_0$), Stage-II ($pT_3N_0M_0$ or $pT_4N_0M_0$), Stage-III (pT_1-$T_2N_1M_0$ or $pT_1N_2M_0$ or pT_3-$T_4N_1M_0$ or pT_2-$T_3N_2M_0$ or $pT_4N_2M_0$), and Stage-IV (any T, any N, M_1). The size of the polyp, its dysplasia, its cytopathological aspect, and the TNM classification were used to define a scale of severity of colorectal lesions in 7 ordinal categories: Severity level-0 (LRP), level -1 (HRP), level-2 (Stage-0), level-3 (Stage-I), level-4 (Stage-II), level-5 (Stage-III), and level-6 (Stage-IV). The main studied factors were the value of $Fhb_{(-1)}$ and the variation of the Fhb between $test_{(-1)}$ and $test_{(0)}$. The value of $Fhb_{(-1)}$ expressed in ngHb/mL (0, 0-50, 50-100, 100-150) and its variation in ngHb/mL (≤ 0, 0-50, 50-100, 100-150, 150-300, ≥ 300) were treated as discrete variables. The cofactors studied were: (1) the participation in at least one gFOBT campaign (new participant: -the person under 50-years-old at the time the gFOBT was used in the program-, No-gFOBT: -the person has never completed a gFOBT despite being often invited to the gFOBT campaigns-, Yes-gFOBT: -the person completed at least one gFOBT campaign-); (2) the age at the time of performing $test_{(-1)}$ (50-54, 55-59, 60-64, 65-69, ≥ 70-years-old); (3) the gender (female *vs* male); (4) the delay (mo) between $test_{(-1)}$ and $test_{(0)}$ (≤ 24, 25-30, > 30) and; (5) the number of tests completed before the $test_{(-1)}$ (0, 1, 2, 3, ≥ 4).

Statistical analysis

The main characteristics were described in frequencies for qualitative variables and in mean ± SD for quantitative variables. Proportions were compared using Pearson Chi-2 test or Fisher's exact test when appropriate.

The relationship between the diagnosis of neoplasia (positive colonoscopy *vs* negative colonoscopy) and the predictive factors [$Fhb_{(-1)}$, participation in at least one gFOBT campaign, age at $test_{(-1)}$, gender, delay between $test_{(-1)}$ and $test_{(0)}$, number of tests completed before $test_{(-1)}$] was analyzed in a multivariate logistic regression model, with the estimation of the adjusted odds ratio (OR) and its 95% confidence interval (CI). For the construction of the multivariate model, all the adjustment covariates regardless of the strength of association in univariate analysis were used. In addition, a strong correlation existed between several covariates [age, delay, number of tests completed before $test_{(-1)}$, participation in at least one gFOBT campaign], the model was extended to the terms of interaction between these covariates. Only the significant interaction terms ($P < 0.05$ in univariate analysis) were retained in the final model evaluated by the likelihood ratio test. Positive tests without colonoscopy at the time of the study ($n = 340$) were excluded from this logistic regression analysis.

The relationship between the severity of the lesions (ordinal variable; 0 to 6) and the predictive factors [$Fhb_{(-1)}$, participation in at least one gFOBT campaign, age at $test_{(-1)}$, gender, delay between $test_{(-1)}$ and $test_{(0)}$, number of tests completed before $test_{(-1)}$] was analyzed in an ordered, multivariate polytomous regression model, with the estimation of the risk and its 95%CI. All the adjustment covariates regardless of the strength of association in univariate analysis were used in the multivariate model. In addition, the model was extended to terms of interaction between covariates with a strong correlation (cited above). Only the significant interaction terms ($P < 0.05$ during a univariate analysis) were retained in the final model evaluated by the likelihood ratio test. In this analysis, the unspecified polyps ($n = 3664$) were classified as the polyps not at risk. Similarly, cancers without any precision (unknown/unspecified, $n = 101$) on the TNM classification were classified as tumors *in situ*. As a reminder, negative colonoscopies ($n = 2846$) were excluded from this ordered, univariate, and multivariate polytomous regression. The differences were significant at the 5% level with version 13 of the STATA software (College Station, TX, United States).

Ethical considerations

Before analysis, all data were anonymized. The screening database had a favorable opinion from the institution that oversees the ethics of data collection ("Commission nationale de l'Informatique et des libertés")[26]. According to the current French

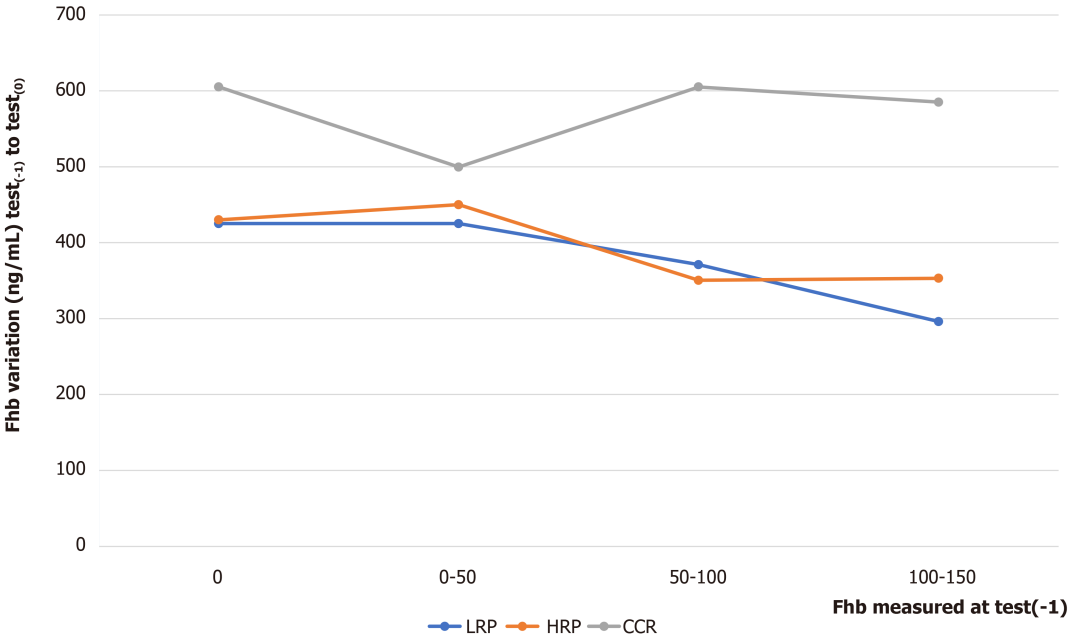


Figure 1 Type of colorectal lesion according to fecal hemoglobin concentration variation (ng/mL) between $test_{(-1)}$ and $test_{(0)}$ and fecal hemoglobin concentration measured at $test_{(-1)}$. Fhb: Fecal hemoglobin; LRP: Low risk polyps; HRP: High risk polyps.

legislation, a study that does not change the care of patients did not require the opinion of the Clinical Research Centers Ethics Committee. This article does not contain any studies with human participants performed by any of the authors. This study does not involve human participants, and an informed consent was therefore not required. This article does not contain any studies with animals performed by any of the authors.

RESULTS

A total of 575587 patients completed at less one FIT between 2015 and 2019. Among them, 281837 (49.0%) people were excluded from this etiological analysis because they had completed only one FIT during the study duration. Finally, our study focused on the 293750 people who completed at least two FIT such as $test_{(-1)}$ and $test_{(0)}$. With a mean age of 61.1 ± 6.7 when completing the $test_{(-1)}$, the positivity rate of the $test_{(0)}$ was 4.0% in these 293750 people. This positivity rate was significantly higher ($P < 0.001$) among patients who have a $Fhb_{(-1)}$ between 100 and 150 ngHb/mL (Table 1).

A total of 282156 people (*i.e.* 96.2% of the sample) had a negative $test_{(0)}$ [$Fhb_{(0)} < 150$ ngHb/mL]. The colonoscopy completion rate was globally estimated at 97.1% in 1594 people who had a positive $test_{(0)}$ [$Fhb_{(0)} \geq 150$ ngHb/mL]. The colonoscopy detection rate was 77.7% among those 11254 people who had a colonoscopy, with a total of 8745 colonic lesions detected (including 2182 LRP, 2400 HRP, 502 CRC). This colonoscopy detection rate varied significantly ($P < 10^{-3}$) with the $Fhb_{(-1)}$ [0: 75.6%, (0-50): 77.3%, (50-100): 88.7%, (100-150): 90.3%; $P < 0.001$) (Table 1).

In total, 27.4% of the lesions detected (*i.e.* 2395 cases) were detected in people who completed a $test_{(0)}$ more than 30 mo after the $test_{(-1)}$. However, these 2395 lesions were mostly polyps compared to the lesions detected in people who completed a $test_{(0)}$ within a reasonable delay between $test_{(0)}$ and $test_{(-1)}$ (delay ≤ 24 m -LRP: 63.8%, HRP: 30.3%, CRC: 5.9%-; delay in 24-30 m -LRP: 64.2%, HRP: 29.3%, CRC: 6.5%-; delay > 30 m -LRP: 74.1%, HRP: 21.9%, CRC: 4.0%-; $P = 0.0001$).

For the same value of the previous Fhb [$Fhb_{(-1)}$], the colonoscopy detection rate was not significantly different between the positive values of $Fhb_{(0)}$ [*i.e.* $Fhb_{(-1)} = 0$ & $Fhb_{(0)} =$ (150-300): rate = 75.5% *vs* $Fhb_{(-1)} = 0$ & $Fhb_{(0)} \geq 300$: rate = 75.7%; $P = 0.8$). However, the colonoscopy detection rate was significantly higher when the $Fhb_{(0)}$ was higher, from 75.6% when $Fhb_{(0)}$ was 0 ngHb/mL to 90.3% at (100-150) ngHb/mL (Table 2).

Regardless of the age group, the colonoscopy detection rate was significantly different between the values of $Fhb_{(-1)}$ [*i.e.* age ≥ 70 years: $Fhb_{(-1)} = 0$: rate = 79.1%, $Fhb_{(-1)} =$ (0-50): Rate = 75.9%, $Fhb_{(-1)} =$ (50-100): Rate = 88.2%, $Fhb_{(-1)} =$ (100-150): Rate = 93.8%;

Table 1 Result of the last test completed, based on the characteristics of the study population

Characteristics	Result of the last test [test$_{(0)}$]														
	Positive test and colonoscopy completion rate			Colonoscopy detection rate		Number of the colorectal lesions									
	Nb. (%T+)	*P* value[1]	Number T+ (% Colo in T+)	Number Colo (% Positive Colo)	*P* value[1]	All lesions	Polyps			CRC					
							LRP	HRP	UP	S-0	S-I	S-II	S-III	S-IV	USC
Overall	293750 (4.0)		11594 (97.1)	11254 (77.7)		8748	2182	2400	3664	133	130	53	64	21	101
Fhb$_{(-1)}$ (ngHb/mL)		$< 10^{-3}$			$< 10^{-3}$										
0	280012 (3.5)		9655 (97.0)	9368 (75.6)		7085	1890	1769	3070	85	99	31	47	15	79
0-50	1680 (10.4)		174 (98.9)	172 (77.3)		133	27	41	56	3	4	1	0	0	1
50–100	8776 (13.0)		1144 (96.9)	1108 (88.7)		983	177	360	370	25	16	12	9	2	12
100-150	3282 (18.9)		621 (97.6)	606 (90.3)		547	88	230	168	20	11	9	8	4	9
gFOBT campaign participation		$< 10^{-3}$			0.03										
Yes-gFOBT	213476 (4.2)		8874 (97.0)	8605 (77.8)		6695	1760	1906	2603	112	109	44	59	17	85
New entrant	40199 (2.9)		1156 (97.5)	1127 (75.1)		846	168	203	456	8	6	0	0	1	4
No-gFOBT	40075 (3.9)		1564 (97.3)	1522 (79.3)		1207	254	291	605	13	15	9	5	3	12
Age (yr) at test$_{(-1)}$		$< 10^{-3}$			$< 10^{-3}$										
50-54	57625 (3.0)		1727 (97.9)	1690 (73.7)		1245	276	348	582	10	14	1	6	1	7
55-59	62489 (3.5)		2166 (97.3)	2107 (75.6)		1592	389	445	687	21	19	4	7	3	17
60-64	64141 (3.9)		2514 (97.4)	2449 (78.4)		1920	509	503	807	28	19	17	15	7	15
65-69	63915 (4.7)		2997 (97.0)	2907 (78.7)		2287	592	647	886	38	54	14	17	5	34
≥ 70	45580 (4.8)		2190 (95.9)	2101 (81.1)		1704	416	457	702	36	24	17	19	5	28
Gender		$< 10^{-3}$			$< 10^{-3}$										
Female	160181 (3.3)		5343 (97.1)	5188 (72.8)		3778	962	937	1663	59	51	15	33	11	47
Male	133569 (4.7)		6251 (97.0)	6066 (81.9)		4970	1220	1,463	2001	74	79	38	31	10	54
Delay (mo) between test$_{(-1)}$ and test$_{(0)}$		$< 10^{-3}$			$< 10^{-3}$										
≤ 24	40422 (5.2)		2111 (94.3)	1991 (72.2)		1437	435	435	482	21	23	12	12	0	17

24-30	163470 (4.0)		6538 (97.7)	6390 (76.9)		4916	1322	1440	1833	99	83	29	39	14	57
> 30	89858 (3.3)		2945 (97.6)	2873 (83.4)		2395	425	525	1349	13	24	12	13	7	27
Nb. of tests completed before test$_{(-1)}$		$< 10^{-3}$			0.01										
0	68839 (3.5)		2421 (97.1)	2351 (76.1)		1790	396	465	855	19	21	9	5	4	16
1	54790 (3.7)		2044 (97.7)	1996 (77.6)		1548	364	432	667	20	26	6	13	6	14
2	55794 (4.0)		2245 (97.1)	2180 (76.6)		1670	434	472	678	25	17	11	12	2	19
3	56716 (4.4)		2514 (97.3)	2445 (77.9)		1905	553	562	649	34	37	13	24	5	28
4	57611 (4.1)		2370 (96.3)	2282 (80.4)		1835	435	469	815	35	29	14	10	4	24

[1]Pearson Chi-2 test or Fisher's exact test. Fhb$_{(0)}$: Fecal hemoglobin measured at test$_{(0)}$ (last screening test); Fhb$_{(-1)}$: Fecal hemoglobin measured at test$_{(-1)}$ (penultimate test); CRC: Colorectal cancer; Colo: Colonoscopy; gFOBT: Guaiac fecal occult blood test; HRP: High risk polyps; LRP: Low risk polyps; UP: Unspecified-polyp; USC: Unspecified stage of colorectal cancer; S-0: Colorectal cancer Stage-0 ($pTisN_0M_0$), S-I: Stage-I ($pT_{1-2}N_0M_0$); S-II: Stage-II ($pT_3N_0M_0$ or $pT_4N_0M_0$); S-III: Stage-III (pT_1-$T_2N_1M_0$ or $pT_1N_2M_0$ or pT_3-$T_4N_1M_0$ or pT_2-$T_3N_2M_0$ or $pT_4N_2M_0$); S-IV: Stage-IV (any T, any N, M_1); T+: Positive test.

P = 0.001). In the age groups of 65-69 years and ≥ 70 years, the proportion of CRC among colorectal lesions was significantly higher when the Fhb$_{(-1)}$ was between 100 and 150 [*i.e.* age in 65-69 years: Fhb$_{(-1)}$ = 0: %CRC = 6.2, Fhb$_{(-1)}$ = (0-50): %CRC = 8.2, Fhb$_{(-1)}$ = (50-100): %CRC = 8.1, Fhb$_{(-1)}$ = (100-150): %CRC = 15.8; P = 0.001) (Table 3).

Overall, the proportion of recto-sigmoid lesions was 57.2% among the 5100 lesions for which the location was provided. The proportion of CRC among these colorectal lesions was significantly higher (P = 0.01) in cecal localization compared to other colonic locations. However, this increase in the proportion of CRC among the cecal lesions was not statistically significant regardless of the stratum combining Fhb$_{(-1)}$ and Fhb$_{(0)}$ (Table 4).

Regardless of the Fhb$_{(-1)}$ modalities [except for modality (0-50)], the variation in Fhb between test$_{(0)}$ and test$_{(-1)}$ was significantly greater when the lesion was CRC colorectal cancer. For this modality (0-50), the number of lesions was relatively lower (CRC = 9, LRP = 27, UP = 56, HRP = 41) (Figure 1).

The multivariate logistic regression included 11254 people who completed a colonoscopy. This analysis shows that people with a Fhb$_{(-1)}$ in (100-150) were 2.9 times more likely to be diagnosed with a colorectal lesion in test$_{(0)}$ compared to those having a Fhb$_{(-1)}$ value of zero (adjusted OR = 2.9; 95%CI: 2.2-3.8). This risk of detecting a neoplastic lesion increased significantly with the age, the gender, the delay between test$_{(-1)}$ and test$_{(0)}$, and the number of tests performed before test$_{(-1)}$ (Table 5).

The ordered polytomous regression included 8748 people having exhaustive information on the colorectal lesion. The analysis showed that people with a Fhb$_{(-1)}$ in (100-150) (P = 0.001) were 2.6 (2.2; 3.0) times more likely to have a high severity level, compared to those having a Fhb$_{(-1)}$ value of zero. The risk to have a high severity level

Table 2 Colonoscopy detection rate, number and severity of colorectal lesions according to the previous rate and the last rate of fecal hemoglobin

$Fhb_{(0)}$ by $Fhb_{(-1)}$			Colonoscopy detection rate		Number of colorectal lesions by Severity									
$Fhb_{(-1)}$ (ngHb/mL)	$Fhb_{(0)}$ (ngHb/mL)	Number (% in subtotal)	Number Colo (%Positive Colo)	*P* value[1]	Nb. of colorectal lesions	Polyps			CRC					
						LRP	HRP	UP	S-0	S-I	S-II	S-III	S-IV	USC
0				0.8										
	0	257589 (92.0)												
	0-50	1805 (0.6)												
	50-100	8275 (3.0)												
	100-149	2688 (1.0)												
	150-300	4903 (1.7)	4778 (75.5)		3609	980	918	1601	35	38	5	6	2	24
	≥ 300	4752 (1.7)	4590 (75.7)		3476	910	851	1469	50	61	26	41	13	55
	Subtotal	280012 (100.0)	9368 (75.6)		7085	1890	1769	3070	85	99	31	47	15	79
0–50				0.3										
	0	1328 (79.1)												
	0-50	30 (1.8)												
	50-100	112 (6.7)												
	100-149	36 (2.1)												
	150-300	86 (5.1)	85 (74.1)		63	17	18	24	1	2	1	0	0	0
	≥ 300	88 (5.2)	87 (80.5)		70	10	23	32	2	2	0	0	0	1
	Subtotal	1680 (100.0)	172 (77.3)		133	27	41	56	3	4	1	0	0	1
50–100				0.7										
	0	6589 (75.1)												
	0-50	106 (1.2)												
	50-100	700 (7.9)												
	100-149	237 (2.7)												
	150-300	540 (6.2)	525 (88.4)		464	97	174	175	7	4	2	2	0	3
	≥ 300	604 (6.9)	583 (89.0)		519	80	186	195	18	12	10	7	2	9
	Subtotal	8776 (100.0)	1108 (88.7)		983	177	360	370	25	16	12	9	2	12

100–149				0.3									
	0	2244 (68.4)											
	0-50	49 (1.5)											
	50-100	259 (7.9)											
	100-149	109 (3.3)											
	150-300	293 (8.9)	286 (88.8)	254	52	103	87	4	2	3	1	0	2
	≥ 300	328 (10.0)	320 (91.6)	293	36	127	81	16	9	6	7	4	7
	Subtotal	3282 (100.0)	606 (90.3)	547	88	230	168	20	11	9	8	4	9

[1]Pearson Chi-2 test or Fisher's exact test. $Fhb_{(0)}$: Fecal hemoglobin measured at $test_{(0)}$ (last screening test); $Fhb_{(-1)}$: Fecal hemoglobin measured at $test_{(-1)}$ (penultimate test); CRC: Colorectal cancer; Colo: Colonoscopy; HRP: High risk polyps; LRP: Low risk polyps; UP: Unspecified-polyp; USC: Unspecified stage of colorectal cancer; S-0: Colorectal cancer Stage-0 ($pTisN_0M_0$), S-I: Stage-I ($pT_{1-2}N_0M_0$); S-II: Stage-II ($pT_3N_0M_0$ or $pT_4N_0M_0$); S-III: Stage-III (pT_1-$T_2N_1M_0$ or $pT_1N_2M_0$ or pT_3-$T_4N_1M_0$ or pT_2-$T_3N_2M_0$ or $pT_4N_2M_0$); S-IV: Stage-IV (any T, any N, M_1).

was higher in male compared to female (P = 0.001). This risk was reduced by 20% in age group 55-59 years compared to age group < 55 years [adjusted OR: 0.8 (0.6; 1.0), P = 0.02] (Table 5).

DISCUSSION

This study showed that the risk of detecting a colorectal lesion during a campaign was proportional to the Fhb observed in the previous test completed. Above all, it highlighted that an increase in the probability of detecting a colorectal neoplasia with a high level of severity was proportional to the Fhb observed in the previous test completed. These probabilities varied with the socio-demographic characteristics, especially with age.

The proportion of positive tests and the colonoscopy detection rate increased proportionally with the $Fhb_{(-1)}$. Furthermore, the detection of colorectal lesions according to the variation in the Fhb between $test_{(-1)}$ and $test_{(0)}$ allows to support an association between the detection of cancerous lesions and the strong Fhb variations between two consecutive tests. These results agree with previous data already described in the literature. Furthermore, the increased risk of detecting a neoplasia during a new screening 2 years after a negative screening test result has already been reported in Ireland[14] and recently in the Ile-de-France region[27]. These authors observed variable proportions of pathologies (advanced adenomas or CRC) during a subsequent campaign and hypothesized that these lesions could have been diagnosed during the previous campaign if the FIT positivity threshold was not relatively high. However, they admitted that lowering the positivity threshold would both allow the

Table 3 Colonoscopy detection rate and proportion of colorectal cancer among colorectal lesions according to the previous rate of fecal hemoglobin and the age at the time of the previous test

$Fhb_{(-1)}$ by age at $test_{(-1)}$		Colonoscopy detection rate and CRC proportion			
		Number Colo (%Positive colo)	*P* value[1]	Number of lesions (%CRC)	*P* value[2]
Age (yr) at $test_{(-1)}$	$Fhb_{(-1)}$ (ngHb/mL)				
Overall		11254 (77.7)		8748 (5.7)	
50-54			$<10^{-3}$		0.7
	0	1471 (71.6)		1053 (3.1)	
	0-50	21 (71.4)		15 (0.0)	
	50-100	130 (90.8)		118 (2.5)	
	100-150	68 (86.8)		59 (5.1)	
	Subtotal	1690 (73.7)		1245 (3.1)	
55-59			$< 10^{-3}$		0.1
	0	1797 (73.7)		1324 (3.9)	
	0-50	31 (67.7)		21 (4.8)	
	50-100	178 (87.1)		155 (7.1)	
	100-150	101 (91.1)		92 (7.6)	
	Subtotal	2107 (75.6)		1592 (4.5)	
60-64			$< 10^{-3}$		0.05
	0	2044 (76.4)		1562 (4.6)	
	0-50	37 (70.3)		26 (7.7)	
	50-100	247 (90.3)		223 (8.1)	
	100-150	121 (90.1)		109 (8.3)	
	Subtotal	2449 (78.4)		1920 (5.3)	
65-69			$< 10^{-3}$		0.001
	0	2373 (76.5)		1815 (6.2)	
	0-50	54 (90.7)		49 (8.2)	
	50-100	308 (88)		271 (8.1)	
	100-150	172 (88.4)		152 (15.8)	
	Subtotal	2907 (78.7)		2287 (7.1)	
≥70			$< 10^{-3}$		0.01
	0	1683 (79.1)		1331 (6.5)	
	0-50	29 (75.9)		22 (9.1)	
	50-100	245 (88.2)		216 (10.2)	
	100-150	144 (93.8)		135 (13.3)	
	Subtotal	2101 (81.1)		1704 (7.6)	

[1]Pearson Chi-2 test.

[2]Fisher's exact test. CRC: Colorectal cancer; Colo: Colonoscopy; $Fhb_{(-1)}$: Fecal hemoglobin measured at $test_{(-1)}$ (penultimate test).

diagnosis of lesions that could be serious only 2 years later and create an unsustainable endoscopy referral burden[14].

The current positivity threshold in the French screening program induces a considerable loss of chance. Indeed, in most countries, the threshold of FIT positivity is chosen in part to adapt to the offer of colonoscopies. The need to readjust this strategy in patients with a Fhb between 100 and 150 ngHb/mL should be assessed[27]. This

Table 4 Number of colorectal lesions and the proportion of colorectal cancer among colorectal lesions by localization, according to the previous rate and the last rate of fecal hemoglobin

$Fhb_{(0)}$ by $Fhb_{(-1)}$		Colorectal lesion number (%Specified localization)	Number of colorectal lesions by localization and proportion of CRC						
			Specified localization: number (% CRC)	Cecum: number (% CRC)	Right colon: number (% CRC)	Transverse colon: number (%CRC)	Left colon: number (%CRC)	Rectosigmoid: number (%CRC)	*P* value[1]
$Fhb_{(-1)}$ (ngHb/mL)	$Fhb_{(0)}$ (ngHb/mL)								
Overall	150-300	4390 (57.0)	2500 (5.4)	195 (6.7)	490 (5.3)	221 (3.2)	252 (3.2)	1342 (6.1)	0.2
	> 300	4358 (59.7)	2600 (13.4)	179 (16.8)	432 (11.8)	176 (11.4)	235 (11.1)	1578 (14.1)	0.3
	Total	8748 (58.3)	5100 (9.5)	374 (11.5)	922 (8.4)	397 (6.8)	487 (7.0)	2920 (10.4)	0.01
0	150-300	3609 (55.7)	2011 (5.3)	154 (7.8)	391 (5.1)	176 (2.8)	200 (3.0)	1090 (5.8)	0.2
	> 300	3476 (58.2)	2022 (12.0)	145 (14.5)	355 (10.1)	139 (8.6)	185 (10.3)	1198 (12.9)	0.3
	Subtotal	7085 (56.9)	4033 (8.6)	299 (11.0)	746 (7.5)	315 (5.4)	385 (6.5)	2288 (9.5)	0.02
0-50	150-300	63 (61.9)	39 (10.3)	2 (0.0)	6 (16.7)	4 (0.0)	8 (12.5)	19 (10.5)	1.0
	> 300	70 (55.7)	39 (12.8)	2 (0.0)	4 (25.0)	2 (0.0)	3 (0.0)	28 (14.3)	0.8
	Subtotal	133 (58.7)	78 (11.5)	4 (0.0)	10 (20)	6 (0.0)	11 (9.1)	47 (12.8)	0.9
50-100	150-300	464 (62.1)	288 (5.6)	24 (0.0)	66 (4.6)	23 (4.4)	23 (0.0)	152 (7.9)	0.5
	> 300	519 (63.0)	327 (17.1)	22 (27.3)	46 (17.4)	21 (23.8)	29 (10.3)	209 (16.3)	0.5
	Subtotal	983 (62.6)	615 (11.7)	46 (13.0)	112 (9.8)	44 (13.6)	52 (5.8)	361 (12.7)	0.6
100-149	150-300	254 (63.8)	162 (6.2)	15 (6.7)	27 (7.4)	18 (5.6)	21 (4.8)	81 (6.2)	1.0
	> 300	293 (72.4)	212 (21.7)	10 (30.0)	27 (22.2)	14 (21.4)	18 (22.2)	143 (21.0)	1.0
	Subtotal	547 (68.4)	374 (15)	25 (16.0)	54 (14.8)	32 (12.5)	39 (12.8)	224 (15.6)	1.0

[1]Pearson Chi-2 test or Fisher's exact test. CRC: Colorectal cancer; $Fhb_{(0)}$: Fecal hemoglobin measured at $test_{(0)}$ (last screening test); $Fhb_{(-1)}$: Fecal hemoglobin measured at $test_{(-1)}$ (penultimate test).

study highlights the need for a strategy taking into account the age of people participating in the screening campaign. The elderly (≥ 70 years) had a high proportion of positive colonoscopy when the Fhb measured in the previous campaign was between 100 and 150 ngHb/mL. Younger people (< 54 years) had a likelihood of having high-severity neoplasia. Given these results, the recommendation to reduce the FIT positivity threshold to 100 ngHb/mL for first-time arrivals and the elderly (≥ 70 years) should attract the attention of the French health authority.

Another alternative would be to reduce the delay between two tests for these first-time participants and the elderly (age ≥ 70 years) from the current 2 years to 1 year. This alternative could have its main justification in the enthusiasm of the elderly towards screening campaigns in France, described in a previous study[28]. The reduction in the time between two tests has the advantage of allowing the recovery of false negative results that a decrease in the positivity threshold cannot recover. Indeed, after finding that the Fhb was less than 4 μgHb/g in 94.0% of false negative individuals, Ibañez-Sanz *et al*[29] concluded that the decrease in the positivity threshold of the FIT does not increase the detection rate of advanced neoplasia but may increase costs and potential adverse effects. Whatever strategy chosen, it should also include the 65-69-age group, which accounts for more than 33.3% of the CRCs detected and presents a significantly increased proportion of CRC when $Fhb_{(-1)}$ was between 100 and 150.

In terms of the location of colorectal lesions, it has been argued that FITs are possibly less effective at detecting lesions located in the proximal colon than distally [30]. Digby *et al*[19] showed that 77.8% of adenomas and 69.2% of cancers were located in the distal colon. The results of this study are consistent with this proximal location. However, in the Ibañez-Sanz study[29], about 60% of the lesions were localized in the proximal colon, while the expected percentage was 30%.

Table 5 Analysis of the relationship between the diagnosis and severity of colorectal lesions and the predictive factors

Predictive factors	Risk analysis of colorectal lesions in a logistic regression model				Risk analysis of the severity of colorectal lesions in an ordered polytomous regression model			
	Univariate		Multivariate		Univariate		Multivariate	
	Unadjusted OR (95%CI)	*P* value[1]	Adjusted OR (95%CI)	*P* value[1]	Unadjusted OR (95%CI)	*P* value[2]	Adjusted OR (95%CI)	*P* value[2]
Fhb_{-1} (ngHb/mL) (Ref.: 0)								
0-50	1.1 (0.8; 1.6)	0.6	1.1 (0.8; 1.6)	0.5	1.4 (1.0; 2.0)	0.1	1.3 (0.9; 1.8)	0.1
50-100	2.5 (2.1; 3.1)	$< 10^{-3}$	2.4 (2.0; 3.0)	$< 10^{-3}$	1.8 (1.6; 2.1)	$< 10^{-3}$	1.8 (1.6; 2.0)	$< 10^{-3}$
100-150	3.0 (2.3; 3.9)	$< 10^{-3}$	2.9 (2.2; 3.8)	$< 10^{-3}$	2.6 (2.2; 3.1)	$< 10^{-3}$	2.6 (2.2; 3.0)	$< 10^{-3}$
gFOBT campaign participation (Ref.: Yes-gFOBT)								
New entrant	0.9 (0.7; 1.0)	0.04	3.3 (2.2; 5.0)	$< 10^{-3}$	0.6 (0.6; 0.8)	$< 10^{-3}$	0.1 (0.1; 0.2)	$< 10^{-3}$
No-gFOBT	1.1 (1.0; 1.2)	0.2	2.8 (1.9; 4.1)	$< 10^{-3}$	0.8 (0.7; 0.9)	$< 10^{-3}$	0.2 (0.1; 0.3)	$< 10^{-3}$
Age (yr) at $test_{(-1)}$ (Ref.: 50-54)								
55-59	1.1 (1.0; 1.3)	0.2	1.3 (1.0; 1.6)	0.04	1.1 (0.9; 1.3)	0.4	0.8 (0.6; 1.0)	0.02
60-64	1.3 (1.1; 1.5)	$< 10^{-3}$	1.4 (1.2; 1.8)	0.001	1.0 (0.9; 1.2)	0.5	0.7 (0.6; 0.9)	0.004
65-69	1.3 (1.1; 1.5)	$< 10^{-3}$	1.4 (1.1; 1.8)	0.002	1.3 (1.1; 1.5)	0.002	0.9 (0.7; 1.1)	0.2
≥ 70	1.5 (1.3; 1.8)	$< 10^{-3}$	1.7 (1.3; 2.1)	$< 10^{-3}$	1.2 (1.0; 1.4)	0.01	0.8 (0.6; 1.0)	0.07
Gender (Ref.: Female)								
Male	1.7 (1.5; 1.9)	$< 10^{-3}$	1.7 (1.5; 1.8)	$< 10^{-3}$	1.2 (1.1; 1.3)	0.002	1.2 (1.1; 1.3)	$< 10^{-3}$
Delay (mo) between $test_{(-1)}$ and $test_0$ (Ref.: < 24)								
24-30	1.3 (1.1; 1.4)	$< 10^{-3}$	1.3 (1.1; 1.5)	$< 10^{-3}$	1.0 (0.9; 1.1)	0.9	1.0 (0.9; 1.1)	0.9
> 30	1.9 (1.7; 2.2)	$< 10^{-3}$	2.1 (1.7; 2.6)	$< 10^{-3}$	0.6 (0.5; 0.7)	$< 10^{-3}$	0.6 (0.5; 0.7)	$< 10^{-3}$
Number of tests completed before $test_{(-1)}$ (Ref.: 0)								
1	1.1 (0.9; 1.2)	0.3	2.7 (1.9; 3.9)	$< 10^{-3}$	1.2 (1.0; 1.4)	0.03	0.3 (0.2; 0.4)	$< 10^{-3}$
2	1.0 (0.9; 1.2)	0.7	2.7 (1.8; 4.1)	$< 10^{-3}$	1.2 (1.0; 1.3)	0.03	0.2 (0.1; 0.3)	$< 10^{-3}$
3	1.1 (1.0; 1.3)	0.1	2.9 (1.9; 4.4)	$< 10^{-3}$	1.4 (1.2; 1.6)	$< 10^{-3}$	0.3 (0.2; 0.4)	$< 10^{-3}$
4	1.3 (1.1; 1.5)	$< 10^{-3}$	3.5 (2.3; 5.2)	$< 10^{-3}$	1.1 (1.0; 1.3)	0.1	0.2 (0.1; 0.3)	$< 10^{-3}$

[1]*P* values were estimated from likelihood ratio tests based on data from 11254 people who completed a colonoscopy.

[2]*P* values were estimated from likelihood ratio tests based on data from 8748 people diagnosed with colorectal lesion after colonoscopy. $Fhb_{(-1)}$: Fecal hemoglobin measured at $test_{(-1)}$ (penultimate test); gFOBT: Guaiac fecal occult blood test; OR: Odds ratio; CI: Confidence interval.

The optimal interval for CRC screening using FIT remains unclear[5]. In terms of the impact of the delay between the tests on the risk of detection of a colorectal lesion, the results of this study are not consistent with Van Roon's analysis, certainly because of the lower test positivity threshold (≥ 50 ng/mL) in their study carried out in a small sample (7501 people)[31]. In addition, this study found an unexpected reverse direction in the analysis of the lesion severity risk according to the delay between $test_{(0)}$ and $test_{(-1)}$. This paradoxical reduction in the risk of severity could be explained by the high proportion of polyps among the lesions detected in patients with an abnormally long delay between $test_{(0)}$ and $test_{(-1)}$. We can also hypothesize that some patients with a time between the two tests greater than 2 years may be symptomatic and therefore will not appear in the screening program as mentioned by Liao *et al*[21].

Limitations of the study

The main limitation of this study is the amount of missing data, especially on the stages of the lesions and their colonic locations. However, this is a consequence inherent to retrospective studies that cannot question the results of this study. In addition, the absence of a cancer registry in the "Auvergne Rhône-Alpes Region" does not allow interval cancers to be included in this study.

CONCLUSION

An increased risk of severity of the colorectal lesion was observed in proportion to the increase in $Fhb_{(-1)}$. This risk of severity varied with the socio-demographic characteristics of the patients, especially among first-time participants. An increased colonoscopy detection rate was observed in the elderly in correlation with the increase in $Fhb_{(-1)}$. According to these results, the FIT positivity threshold should be reduced to 100 ngHb/mL for first-time participants and patients aged ≥ 70. The other alternative should be to reduce the delay between the two tests for these first-time participants and the elderly (age ≥ 70) from the current 2 years to 1 year.

ARTICLE HIGHLIGHTS

Research background

The rate of positive tests using fecal immunochemical test (FIT) does not decrease with subsequent campaigns, but the positive predictive value of advanced neoplasia significantly decreases in subsequent campaign after a first negative test. A relationship between the fecal hemoglobin concentration (Fhb) and the opportunity to detect a colorectal cancer in subsequent campaign has been shown.

Research motivation

In this period of implementation of the optimization strategies of the French program, our motivation was to alert the health authority on the severity of the lesions not diagnosed because of the high positivity threshold of the current screening FIT.

Research objectives

Our objective was to predict the severity of colorectal lesions based on Fhb measured during previous colorectal cancer screening campaign.

Research methods

The etiological study included 293750 patients aged 50-74, living in Auvergne-Rhône-Alpes (France). These patients completed at least two FIT [$test_{(-1)}$ and $test_{(0)}$] between June 2015 and December 2019. Delay between $test_{(-1)}$ and $test_{(0)}$ was > 1 year, and $test_{(-1)}$ result was negative (< 150 ngHb/mL). The severity of colorectal lesions diagnosed at $test_{(0)}$ was described according to Fhb measured at $test_{(-1)}$ [$Fhb_{(-1)}$]. The relationship between the severity classified in seven ordinal categories and the predictive factors was analyzed in an ordered multivariate polytomous regression model.

Research results

The $test_{(0)}$ positive rate was 4.0% and the colonoscopy completion rate was 97.1% in 11594 patients who showed a positive $test_{(0)}$. The colonoscopy detection rate was 77.7% in those 11254 patients who underwent a colonoscopy. In total, 8748 colorectal lesions were detected (including 2182 low-risk-polyps, 2400 high-risk-polyp and 502 colorectal cancer). The colonoscopy detection rate varied significantly with $Fhb_{(-1)}$ [0 ngHb/mL: 75.6%, (0-50): 77.3%, (50-100): 88.7%, (100-150): 90.3%; P = 0.001). People with a $Fhb_{(-1)}$ within (100-150 ngHb/mL) (P = 0.001) were 2.6 (2.2; 3.0) times more likely to have a high severity level compared to those having a $Fhb_{(-1)}$ value of zero. This severity risk was reduced by 20% in patients aged 55-59 compared to those aged < 55 [adjusted odds ratio: 0.8 (0.6; 1.0)].

Research conclusions

The study showed that higher $Fhb_{(-1)}$ is correlated to an increased risk of severity of colorectal lesions. This risk of severity increased among first-time participants (age < 55) and the elderly (≥ 70). To avoid the loss of chance in these age groups, the FIT

positivity threshold should be reduced to 100 ngHb/mL. The other alternative would be to reduce the time between the two tests in these age groups from the current 2 years to 1 year.

Research perspectives

At the end of this study, we aim to conduct an experiment with a screening program considering the age of patients and the previous values of the fecal hemoglobin concentration.

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CASE REPORT

Plexiform angiomyxoid myofibroblastic tumor treated by endoscopic submucosal dissection: A case report and review of the literature

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Author contributions: Wu JD reviewed the literature and contributed to manuscript drafting; Xu FH and Chen YX were the patient's primary doctors and substantially contributed to the conception and design of the study and acquisition of the data; Luo C and Zhang L substantially contributed to the data acquisition; Hou XH and Song J were responsible for the revision of the manuscript for important intellectual content.

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Abstract

BACKGROUND

Plexiform angiomyxoid myofibroblastic tumor (PAMT) is a rare mesenchymal tumor characterized by multiple nodular plexiform growth patterns and an immunophenotype with myofibroblasts. The pathological characteristics, immunohistochemistry, diagnostic criteria, differential diagnosis, and gene-level changes of PAMT have been reported in many studies. At present, the main treatment for PAMT in the reported cases is surgery; only eight cases were treated *via* endoscopy (excluding 1 thoracoscopic resection), and the lesions were all smaller than 5 cm. There are no reports on the prognosis and follow-up of young patients with lesion sizes reaching 5 cm who undergo endoscopic submucosal dissection (ESD). Herein, we present the first case of a young patient with a lesion size reaching 5 cm who was diagnosed with PAMT *via* endoscopic submucosal dissection.

CASE SUMMARY

A 15-year-old young man with upper abdominal pain for 2 years presented to the Gastroenterology Department of our hospital. Painless gastroscopy showed a semicircular bulge approximately 5 cm in size in the lesser curvature near the cardia of the fundus; the surface was eroded, and shallow ulcers had formed. The pathological manifestations of the biopsy were spindle cell proliferative lesions with interstitial mucinous changes, and the surface mucosa showed chronic inflammatory changes with active lesions; immunohistochemistry showed smooth muscle actin (SMA) (+), CD117 (-), CD34 (-), DOG-1 (-), S-100 (-), and Ki67 (LI: < 1%). We performed ESD on the patient. The lesion that we removed was 5 cm × 4 cm × 2 cm in size. Pathologically, the resected tissue displayed typical manifestations, such as fat spindle-shaped fibroblasts and myofibroblast-like cells showing irregular nodular hyperplasia. Immunohistochemistry staining of the tumor cells revealed the following: CD34 (partially +), SMA (weakly +), CD117 (-), DOG-1 (-),

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S-100 (-), SDHB (+), PCK (-), and Ki67 (labelling index: 2%). There was no recurrence or metastasis during the 3-mo follow-up after the operation, and the treatment effect was good. We also performed a review of the literature on the clinical manifestations, pathological features, immunohistochemistry, and differential diagnosis of PAMT.

CONCLUSION

At present, the diagnostic criteria for PAMT are relatively clear, but the pathogenesis and genetic changes require further study. PAMT is benign in nature, and these patients are less likely to experience local or metastatic recurrence. The main treatment is still surgery if the lesion is in the stomach. Partial gastrectomy and distal gastrectomy are the most frequently performed surgical treatments for PAMT, followed by local resection, subtotal gastrectomy, and wedge resection. But for comprehensive evaluation of the disease, ESD can be considered a suitable method to avoid excessive treatment.

Key Words: Plexiform angiomyxoid myofibroblastic tumor; Endoscopic submucosal dissection; Stomach; Cardia-preserving; Benign; Case report

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Core Tip: Plexiform angiomyxoid myofibroblastic tumor (PAMT) is a rare mesenchymal tumor. The main treatment for PAMT in the reported cases is surgery. We present a rare case of PAMT in a young patient who underwent endoscopic submucosal dissection (ESD). The biopsied and resected tissue showed interstitial mucinous changes and myofibroblast-like cells showing irregular nodular hyperplasia, and immunohistochemistry revealed that the tissue was SMA (+). There was no recurrence during the 3-mo follow-up after ESD, and the treatment effect was good. For comprehensive evaluation of the disease, ESD can be considered a suitable method to avoid excessive treatment.

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INTRODUCTION

Plexiform angiomyxoid myofibroblastic tumor (PAMT), also termed plexiform fibromyxoma (PF), is a rare benign tumor of the gastrointestinal tract that was first proposed by Takahashi *et al*[1] in 2007. This tumor occurs mainly in the antrum of the stomach and shows unique histological features, including myxoid extracellular matrix and ovoid or spindle cells as a plexiform intramural growth pattern. Tumor cells show differentiation into myofibroblasts with SMA expression and have limited cytological atypia[2]. Although some reports showed extragastric expansion and vascular infiltration, no local metastasis or distant recurrence after resection was reported. The main treatment for PAMT in the reported cases is surgery. Herein, we present the first case of a young patient with a lesion size reaching 5 cm who was diagnosed with PAMT *via* endoscopic submucosal dissection.

CASE PRESENTATION

Chief complaints

A 15-year-old patient arrived at the Gastroenterology Department of our hospital complaining of abdominal pain for 2 years.

History of present illness

The patient had intermittent abdominal pain over the past 2 years without special treatment.

History of past illness

The patient had no previous medical history.

Personal and family history

The patient never smoked or drank. He had no history of food or drug allergy and no family history of gastrointestinal tumors.

Physical examination

The temperature of the patient was 36.5 °C, heart rate was 73 beats per minute, breathing rate was 16 beats per minute, and blood pressure was 130/79 mmHg. Examination of the chest, lung, and abdomen showed no abnormalities.

Laboratory examinations

The patient's alkaline phosphatase level was 197 U/L, glutamyl transfer peptidase was 6 U/L, uric acid was 431.6 μmol/L, and phosphorus was 1.41 mmol/L. Other routine blood tests, routine urine tests, routine stool tests, and biochemical and erythrocyte sedimentation rates were all normal.

Imaging examinations

Computed tomography (CT) of the lungs and abdomen revealed no obvious abnormalities in both the lungs and mediastinum, but a space-occupying lesion in the gastric fundus area. Our clinical diagnostic consideration was gastric stromal tumor.

Further diagnostic work-up

Painless gastroscopy showed a semicircular bulge approximately 5 cm in size in the lesser curvature near the cardia of the fundus; the surface was eroded, and shallow ulcers had formed. A biopsy sample was taken from the surface of the lesion (Figure 1). Endoscopic ultrasonography revealed that the lesion originated from the submucosal layer, which was heterogeneous and hyperechoic, and the posterior muscularis propria and serosal surface were present. The blood supply to the lesion was abundant by Doppler ultrasound (Figure 2). Pathologically, the biopsy sample revealed spindle cell proliferative lesions with interstitial mucinous changes, and the surface mucosa showed chronic inflammatory changes with active lesions (Figure 3). Immunohistochemistry showed smooth muscle actin (SMA) (+), CD117 (-), CD34 (-), DOG-1 (-), S-100 (-), and Ki67 (labelling index: < 1%).

FINAL DIAGNOSIS

Based on the pathological results and clinical results, the final diagnosis was PAMT of the stomach.

TREATMENT

Because the lesion was near the cardiac area and the patient was young, we decided to remove the lesion by endoscopy. We performed ESD of the lesion, and as expected, the operation procedure was very difficult. The method of ESD was described everywhere. Simply, we marked the extent of the lesion first. Then, we performed submucosal injections and incised the mucosa at the outer edge of the lesion with a Dual knife and IT knife. After separating the submucosa, the lesion was exposed and dissected with the aid of the snare traction. After nearly 6 h of effort, we resected the lesion successfully (Figure 4A). The blood vessels of the lesion were treated with hemostatic agents during and after the operation. There was no active bleeding or perforation after washing. The cardia was preserved, and the lesion that we removed was 5 cm × 4 cm × 2 cm in size (Figure 4B).

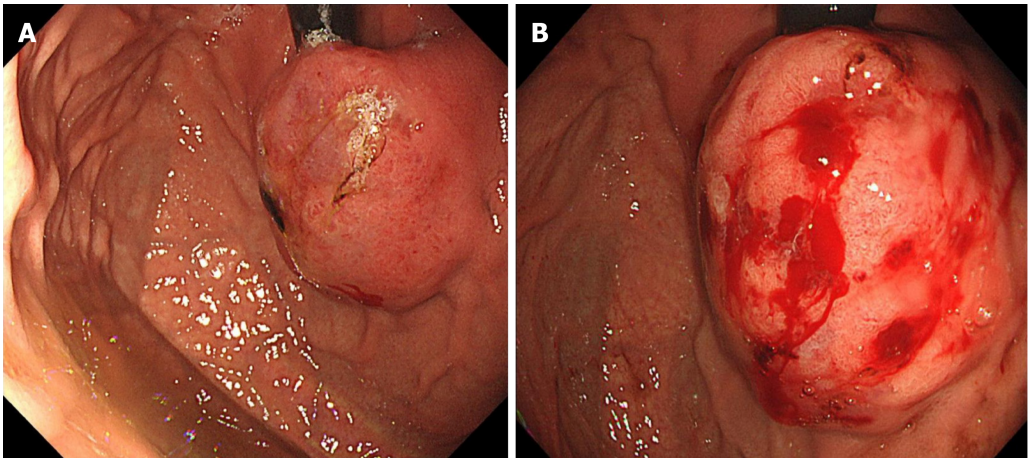


Figure 1 Lesions under endoscopy before and after biopsy. A: Image of the lesion before biopsy; B: Image of the lesion after biopsy.

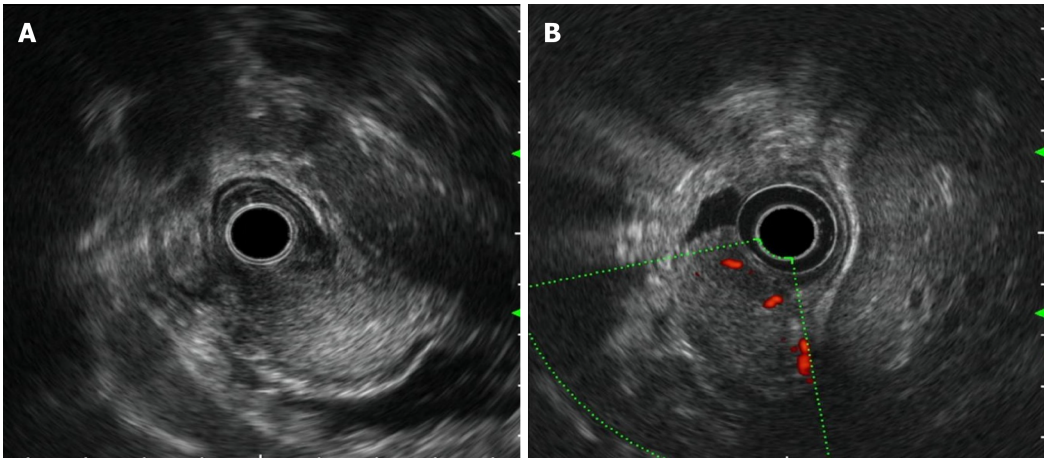


Figure 2 Rich blood flow in the lesion under Doppler ultrasound. A: Endoscopic ultrasonography revealed that the lesion originated from the submucosal layer, which was heterogeneous and hyperechoic, and the posterior muscularis propria and serosal surface were present; B: The blood flow was abundant on Doppler ultrasound.

OUTCOME AND FOLLOW-UP

The patient had sudden abdominal pain on the second day after the operation, and the upper abdomen showed signs of abdominal muscle tension, tenderness, and rebound pain. The results of emergency CT examination showed perforation of the gastrointestinal tract and abdominal effusion. The patient was treated by gastrointestinal decompression, anti-inflammatory therapy, acid suppression, fluid rehydration, and pain relief. On the third day after the operation, the patient was accompanied by fever, and the abdominal symptoms and peritoneal irritation did not improve. Therefore, the patient underwent laparoscopic gastric perforation repair, intestinal adhesion release, and abdominal drainage operation. During the operation, there was a 1-cm perforation in the lower anterior wall of the gastric cardia, and the surrounding tissues were congested and edema. A large abscess cavity formed with the spleen and the left lobe of the liver under the diaphragm. More purulent fluid can be seen in the liver, right paracolic sulcus, intestines, and pelvis. We cleaned the abdominal cavity, sutured the perforation, and strengthened the seromuscular layer. The patient fully recovered without any symptoms and was discharged after 14 d. There was no recurrence during the 3-mo follow-up after ESD (Figure 4C). The pathology of the resected tissue showed typical manifestations, such as abundant slender blood vessels, rich mucus-like mesenchyme, and spindle-shaped, fat spindle-shaped fibroblasts and myofibroblast-like cells showing irregular nodular hyperplasia (Figure 5). Immunohistochemistry staining of tumor cells revealed the following: CD34 (partially +), SMA (weakly +), CD117 (-), DOG-1 (-), S-100 (-), SDHB (+), PCK (-), and Ki67 (labelling index: 2%).

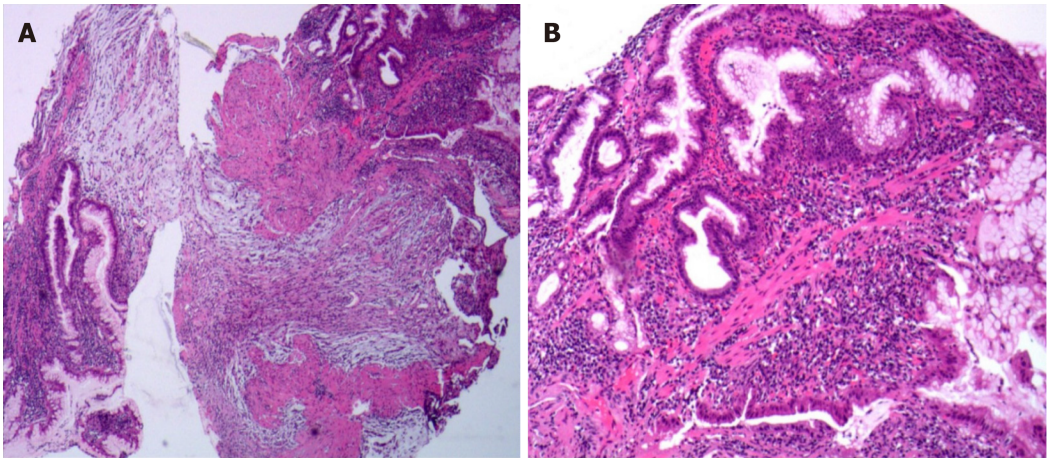


Figure 3 Hematoxylin and eosin staining of the biopsy revealed spindle cell proliferative lesions with interstitial mucinous changes, and the surface mucosa showed chronic inflammatory changes with active lesions. A: Magnification: 10 ×; B: Magnification: 40 ×.

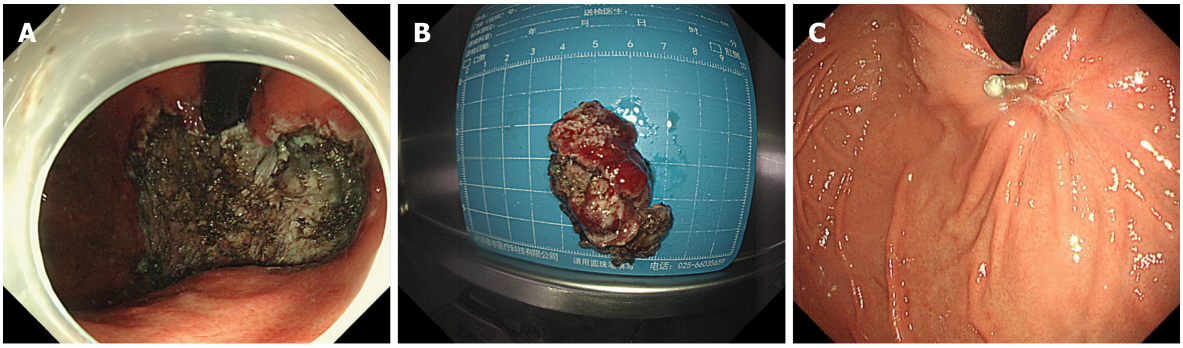


Figure 4 Images of peeled tissue, lesion size, and clipping under endoscopy. A: The wound after endoscopic submucosal dissection (ESD); B: Resected tissue: 5 cm × 4 cm × 2 cm; C: The ulcer scar of the wound 3 mo after ESD.

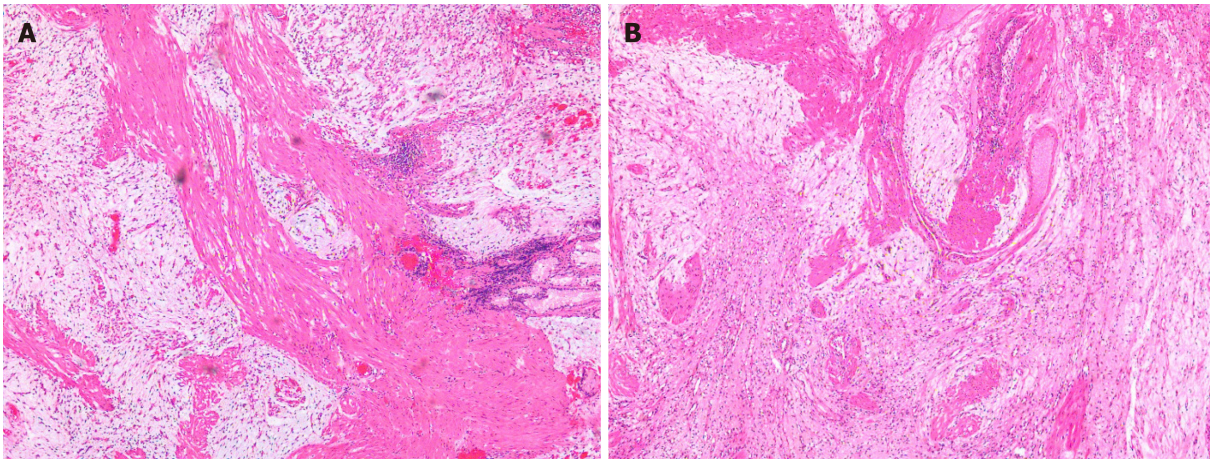


Figure 5 Abundant slender blood vessels, rich mucus-like mesenchyme and spindle-shaped, fat spindle-shaped fibroblasts, and myofibroblast-like cells showing irregular nodular hyperplasia. A: Magnification: 20 ×; B: Magnification: 10 ×.

DISCUSSION

In 2007, Takahashi *et al*[1] first reported a rare gastric mesenchymal tissue tumor and named it PAMT[1,4]. Miettinen *et al*[3] first coined the term PF for this type of lesion in 2009, and subsequently, the term was adopted in WHO 2010 classification of digestive system tumors in the chapter "Mesenchymal Tumours of the Stomach," which was wrote in collaboration with Miettinen[23]. The term PF was used due to its cellular

architecture and fibromyxoid nature, allowing nonprofessionals to recognize that it is a benign disease when encountering this spectrum of lesions. Despite that the WHO classification established the nomenclature, many authors still believe that the term PAMT better describes the histogenesis and histology of such tumors[16,26,27].

Since then, until 2019, only 121 additional cases have been published in the literature[5], and two more cases have been added in 2020. Miettinen *et al*[3] reported that the incidence of gastric PAMT is very low, less than 1/150 of gastrointestinal stromal tumors (GISTs). The range in patient ages is broad, from 5 to 81 years old (average age, 43.17 ± 18.00 years; median age, 46 years). The patients were mostly middle-aged and elderly people, and the peak was usually between 30 and 60 years old[4]. Lesions are frequently located in the gastric antrum but occasionally located in the small intestine, other parts of the stomach, large intestine, mediastinum, and esophagus[24]. According to a recent review report, the maximum diameter of the tumor ranged from 0.8-17 cm, with a mean size of 4.81 ± 3.30 cm and median size of 4.0 cm[4].

Although PAMT is benign, it shows extensive vascular proliferation; therefore, clinical manifestations can be accidental discovery, general gastrointestinal symptoms, and gastrointestinal bleeding. Typical PAMT manifests as general gastrointestinal symptoms, including abdominal pain, abdominal distension, indigestion, loss of appetite, heartburn, diarrhea, and epigastric discomfort. Manifestations of gastrointestinal bleeding are also common and can lead to gastrointestinal bleeding-related manifestations such as anemia, melena, and hematemesis[6-9]. Some patients experience dull upper abdominal pain, along with the presence of a lump in the right hypochondrium[24].

The currently reported cases of PAMT have unique characteristics: (1) The tumor can be polypoid, nodular, or cystic. PAMT forms a leaf-like intramural/submucosal mass, which is light pink, brown, or red in color, usually shiny and mucinous, and very hard. The margins usually have a good outline, such as polypoid, nodular, and cystic lesions, but they are not completely different[1,3,6,13-16,21,25]. The mass is usually visible endoscopically[5,16,21,25] and radiologically[14,21,25]. The tumor originates from the submucosa or muscularis propria and can infiltrate into other tissue layers, protruding from the mucosa or serous membrane. The surface can be accompanied by ulcers and erosions; (2) histologically, tumor tissue is mainly composed of bland ovoid to spindle-shaped fibroblast-like cells, abundant thin-walled blood vessels, and fibrous mucous matrix. Tumor cells are arranged in an irregular plexiform or multinodular pattern, and are separated by the abundant mucus-like or mucous-fibrous matrix around the small blood vessels. These features are helpful to differentiate PF from other gastrointestinal mesenchymal tumors, such as myxoid GISTs, but further differentiation and confirmation of the diagnosis can be identified by immunostaining[3]. The myxoid matrix is always positive for Alcian blue. The tumor cells show monomorphous oval nuclei containing fuzzy nucleoli and fine chromatin, surrounded by mild eosinophilic cytoplasm with indistinct boundary. Nucleolus and chromatin are delicate and fuzzy[4]. Some cases showed vascular and lymphatic infiltration[3,28], but metastasis or recurrence did not occur after surgery; and (3) in immunohistochemistry and special staining of PAMT, vimentin, SMA, muscle-specific actin, and Alcian blue are generally positive[10], indicating the fibroblastic, myofibroblastic, and smooth-muscle-cell characteristics of PAMT. Current research reports show that vimentin, SMA, and MSA have a high sensitivity and specificity for the diagnosis of PAMT, mainly because they are important markers of smooth muscle tumors and mesenchymal tissues. The immunochemical staining indicators in some specimens such as H-caldesmon, calponin, and desmin were partially positive[1,3,11-17], suggesting possible myofibroblastic differentiation, while these stains have been negative in other specimens[1,3,18-21]. For the muscular lineage toward terminal muscle cell differentiation, desmin and caldesmon are more specific markers. These markers show limited and focal reactive results in PAMT, which is consistent with the myofibroblastic spectrum of PF cell development[4], but they are also expressed in desmoid-type fibromatosis. Equivocal results were found for estrogen receptor (ER) and progesterone receptor (PR) staining. Some case reports have shown that specimens are focally or diffusely positive for PR but negative for ER. Thus, hormone therapy is also a direction to consider, but it may be of little significance[24]. For c-kit, pancytokeratin, S-100 protein, B-catenin, neurofilament, cytokeratin, DOG-1, CD34, epithelial membrane antigen, and ALK-1, all reported cases have negative results[10]. Ki-67 staining commonly illustrates very low proliferation rates, mostly < 2%. CD34 is not usually expressed in leiomyomas or desmoid-type fibromatoses; however, it is normally expressed in endothelial cells, embryonic cells of the hematopoietic system, and solitary fibrous tumors. S100 protein can be expressed

in spindle cell neoplasms of the gastrointestinal (GI) tract, a characteristic of schwannomas. Combination of these markers can help make a differential diagnosis of spindle cell tumors in the GI tract[25]. B-catenin and ALK-1 are always negative in PAMT. CD117 or DOG1 is usually negative in PAMT but positive in GISTs, which do not show a unique plexiform wall growth pattern; thus, it can be separated from PAMT.

The diagnosis of PAMT depends on pathological and immunochemical examinations, and typical microscopic manifestations under high magnification should be observed, including bland ovoid to spindle-shaped fibroblast-like cells, abundant thin-walled blood vessels, and fibrous mucous matrix. Clinicians generally speculate that PAMT is benign based on the limited atypicality of tumor cells and low mitotic rate [22]. Vimentin, SMA, and muscle-specific actin were at least partially positive; Alcian blue was positive; and pancytokeratin, c-kit, DOG-1, CD34, S-100 protein, B-catenin, and ALK-1 were negative[4]. In our case, the tumor was positive for SMA, partly positive for CD34, and negative for DOG-1, S-100, PCK, and Ki67 (labelling index: 2%), which immunohistochemically confirmed PAMT.

For genes, mutations in *c-kit* and *PDGFRA* genes are important and characteristic in GISTs[29], but no studies have reported mutations in these two genes in PAMT. Therefore, PAMT can be further distinguished from GISTs in terms of gene mutations. Some cases have been described as having a repeated translocation, t(11;12)(q11;q13), involving the long noncoding gene metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) and the gene glioma-associated oncogene homologue 1 (*GLI1*). Through repeated *MALAT1-GLI1* translocation or *GLI1* upregulation, *GLI1* overexpression depicts a different pathogenic subgroup of plexiform fibromyxomas with activation of the Sonic Hedgehog signaling pathway. Inappropriate reactivation of the Hedgehog signaling pathway is responsible for the formation and progression of several cancers[30]. In addition to the canonical pathway of Hedgehog signaling, some canonical pathways that may be important for the development of gastrointestinal tumors has been recently described, including a noncanonical, Patched-dependent, and Smoothened-independent pathway[37]. Base on the molecular pathway of PF development, neoplasms with GLI1 oncogenesis may be sensitive to Hedgehog pathway inhibitors, targeting either Patched, Smoothened, GLI1, or other Hedgehog pathway components[31].

For the differential diagnosis of myxoid mesenchymal tumors in the gastrointestinal tract, the main considerations include GIST, leiomyomata, solitary fibrous tumor, leiomyosarcoma (LMS), desmoid tumor, inflammatory fibroid polyp, neurilemmoma, inflammatory myofibroblastic tumor, low-grade fibromyxoid sarcoma, Plexiform nerve fibroma, myxoma and, in women, myxoid low-grade endometrial stromal sarcoma[1,3,11-13,32]. PF is positive for SMA, DES, and vimentin and negative for c-kit, CD34, S-100, DOG1, and CD117. However, GIST may have CD117, CD34, and DOG-1 immunoreactivity and c-kit or PDGFRA mutation. Leiomyoma can be differentiated by the bundle like and braided arrangement of tumor tissue and the eosinophilic cytoplasm of fusiform tumor cells. SMA, MSA, desmin, and caldesmon were positive in leiomyoma, but desmin was negative in PAMT. Myxoid and leiomyoma have positive reaction for SMA, desmin, and caldesmon, and Plexiform neurofibroma have positive reaction for S-100 protein. Solitary fibrous tumors are positive for CD34, and consist of altered hypercellular and hypocellular areas, deposits of dense keloid-type collagen, and hemangiopericytoma-like areas[25].

The main treatment for PAMT is surgery. Current literature reviews up to 2019 reporting on 121 patients demonstrated no local or metastatic reoccurrence among the 84 patients who were followed[4,5]. Some reports also presented cases of PAMT treated *via* endoscopy, but the lesions were smaller than 5 cm[20,33-36]. Our case is the ninth case of endoscopic resection, and it is the largest mass that has been removed endoscopically so far. The lesion reached 5 cm × 4 cm × 2 cm in size and was near the cardia. The diseased tissue was completely removed, and the cardia was preserved. Our patient had no recurrence or metastasis during the 3-mo follow-up after the operation, and the treatment effect was good.

CONCLUSION

At present, the diagnostic criteria for PAMT are relatively clear, but the pathogenesis and genetic changes require further study. PAMT is benign in nature, and these patients are less likely to experience local or metastatic recurrence. The main treatment is still surgery, and when the lesion is in the stomach, partial gastrectomy and distal

gastrectomy are the most commonly performed surgical treatments for PAMT, followed by local gastrectomy, subtotal gastrectomy, and wedge-shaped gastrectomy. But for comprehensive evaluation of the disease, ESD can be considered a suitable method to avoid excessive treatment.

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OPINION REVIEW

Management of Flood syndrome: What can we do better?

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Abstract

Approximately 20% of cirrhotic patients with ascites develop umbilical herniation. These patients usually suffer from multisystemic complications of cirrhosis, have a significantly higher risk of infection, and require accurate surveillance–especially in the context of the coronavirus disease 2019 pandemic. The rupture of an umbilical hernia, is an uncommon, life-threatening complication of large-volume ascites and end-stage liver disease resulting in spontaneous paracentesis, also known as Flood syndrome. Flood syndrome remains a challenging condition for clinicians, as recommendations for its management are lacking, and the available evidence for the best treatment approach remains controversial. In this paper, four key questions are addressed regarding the management and prevention of Flood syndrome: (1) Which is the best treatment approach–conservative treatment or urgent surgery? (2) How can we establish the individual risk for herniation and possible hernia rupture in cirrhotic patients? (3) How can we prevent umbilical hernia ruptures? And (4) How can we manage these patients in the conditions created by the coronavirus disease 2019 pandemic?

Key Words: Umbilical hernia rupture; Ascites; Cirrhosis; Flood syndrome; COVID-19; Literature review

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Core Tip: Flood syndrome is a rare, life-threatening complication of large-volume ascites and end-stage liver disease resulting in a sudden umbilical hernia rupture and spontaneous paracentesis. It remains a challenge for clinicians, as recommendations for the management of this syndrome are lacking. The establishment of the individual risk for herniation and possible hernia rupture, timely prevention, and elective surgical treatment might reduce the risk of complications and the need for urgent surgery.

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INTRODUCTION

Ascites is one of the most common complications of liver cirrhosis, manifesting to a variable extent in over 50% of cases. It is also one of the signs of decompensated cirrhosis that is associated with a poor prognosis[1,2]. The occurrence of ascites impairs patients' working and social lives, leads to more frequent hospitalization, requires chronic treatment, and is a direct cause of further complications–such as spontaneous bacterial peritonitis, restrictive ventilatory dysfunction, and abdominal hernias[2]. The incidence of an umbilical hernia (UH) in cirrhotic patients with ascites is approximately 20%, which is 10 times higher than in the general population, and may be up to 40% in cases of large-volume ascites[3-6].

In rare cases, patients with large-volume ascites develop the severe complication of a ruptured UH, resulting in the leakage of ascitic fluid through a skin lesion (Figure 1). This is also known as Flood syndrome, and it was first described by Frank B. Flood in 1961[5,7,8]. This is a life-threatening complication with a significant morbidity and mortality rate of 30%. The rupture of the UH is contributed to by local trauma or a sudden rise in intraabdominal pressure caused by coughing, vomiting, straining, increasing ascites, or heavy lifting[9,10].

Possible complications of the ruptured UH are the evisceration of the small intestine, the incarceration of the bowel, cellulitis, peritonitis, sepsis, and hypotension caused by massive paracentesis[11,12]. The management of Flood syndrome is the subject of much debate due to the lack of high-quality prospective studies and the absence of clear recommendations.

WHICH IS THE BEST TREATMENT APPROACH–CONSERVATIVE TREATMENT OR URGENT SURGERY?

Mortality rates after urgent surgical repair of the hernia vary between 6%–20%, compared to 60%–80% after only supportive care[5,13-15]. The stabilization of the patient's condition, local wound care, optimal control of ascites, correction of the electrolyte imbalance, coagulopathy and thrombocytopenia, and antimicrobial prophylaxis are the critical points for managing a ruptured UH[5,13,16]. The timing of the surgery is also contested, as there is no reliable data on the optimal duration of conservative treatment. The surgical procedure is often delayed due to the high incidence of postoperative complications, such as wound dehiscence and evisceration caused by the re-accumulation of the ascites, bleeding due to coagulopathy, and the inadequate synthesis of clotting factors[5]. The key points of the management of Flood syndrome are summarized in Figure 2.

Pre-operative and surgical management

Evaluation: Patients with liver disease are at a higher risk of surgical and anesthesia-related complications. This risk depends upon the type of liver disease and its severity, the surgical procedure, and the type of anesthesia[17]. Both the model for end-stage liver disease (MELD) score and the Child-Turcotte-Pugh (CTP) classification, combined with the American Society of Anesthesiologists Classification, have been



Figure 1 Flood syndrome. Infected ulceration of the ruptured umbilical hernia in a patient with ascites and liver cirrhosis (personal archives).

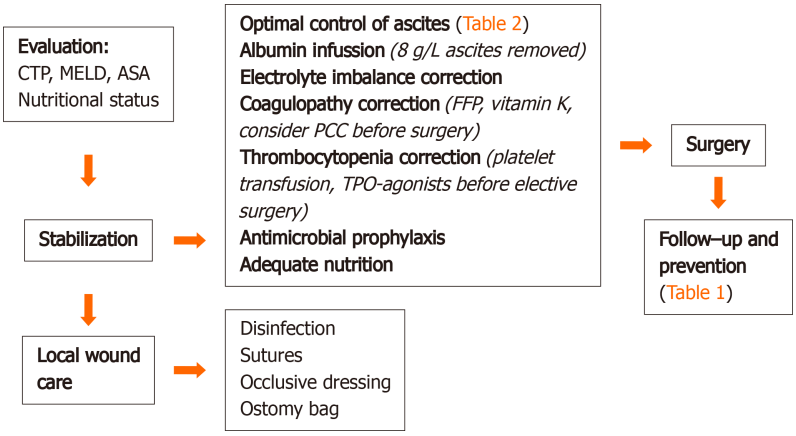


Figure 2 Management of Flood syndrome. ASA: American Society of Anesthesiologists; CTP: Child-Turcotte-Pugh; FFP: Fresh frozen plasma; MELD: Model for end-stage liver disease; PCC: Prothrombin complex concentrate; TPO: Thrombopoietin.

suggested for the stratification of the risks of hernia surgery, but neither appear to be ubiquitously applicable[17]. The main predictor of operative risk in patients with cirrhosis is the CTP classification, but more recent studies suggest that the MELD score might be superior in this regard[18]. Patients with a CTP score > 10 or a MELD score > 20 pose a high risk of postoperative decompensation, including liver failure, worsening encephalopathy, bleeding, wound infection, renal failure, hypoxia, intractable ascites, UH recurrence, and prolonged ileus[5,17,19]. Therefore, it is important to stabilize the patient's condition and to initiate conservative treatment prior to surgery.

Local wound care: Local wound care is one of the first steps in the management of Flood syndrome. Proper disinfection of the area around the skin lesion and control of the peritoneal fluid leakage are crucial in preventing infection and excessive ascites loss. Massive spontaneous paracentesis may lead to severe electrolyte disbalance and paracentesis-induced circulatory dysfunction. Macerations at the UH site might be addressed with two Z sutures and covered with an occlusive dressing. An ostomy bag is used to collect the remaining ascitic fluid.

Stabilization: The optimal control of ascites is one of the main goals in managing a ruptured UH and preventing herniation. Inadequately controlled ascites increase the risk of wound infection and the relapse of the hernia by 75%[5,14]. The main strategies of controlling ascites according to the European Association for the Study of the Liver guidelines are presented in Table 1.

Plasma volume expansion therapy with albumin infusion (8 g/L ascites removed) is recommended to prevent paracentesis-induced circulatory dysfunction in cases with a high volume (5 L of ascites) of spontaneous paracentesis[2]. There is also some promising data regarding cheaper alternative preventive measures, such as colloids,

Table 1 Risk for herniation and possible hernia rupture establishment and prevention of umbilical hernia rupture/reoccurrence

Risk establishment	Prevention
Questions to ask	
(1) Ascites control: (a) Changes in the abdomen volume; (b) Fluid balance; (c) Weight; (d) Use of prescribed treatment; (2) Nutrition (any signs of malnutrition?); (3) Alcohol intake; (4) Surgeries in the abdomen; (5) Pre-existing hernias; (6) Employment (heavy lifting activities); (7) Comorbidities; (8) Constipation; (9) Medicaments used; and (10) Changes in the abdomen, umbilical area visual appearance	(1) Education; (2) Risk establishment; and (3) Risk management: (a) Lifestyle modification; (b) Management of the underlying liver disease; (c) Management of ascites; (d) Doctor-patient communication; and (e) Communication between medical specialists
	Patients with UH
	(1) All the above; (2) Avoid heavy lifting, rapid movement; (3)Abdominal surgeon consult; and (4) Elective surgery in stable patients

UH: Umbilical hernia.

vasoconstrictors (midodrine, terlipressin), and lowering the standard doses of the albumin, but these approaches are not yet recommended by international guidelines [20-23].

It is also essential to evaluate the patient's nutritional status, as these patients are often cachectic and malnourished. A protein-rich diet (2000 kcal/d, with 40–50 g of protein) is recommended to address the patient's nutritional needs. The patient's dietary needs might be covered orally with additional protein supplementation or *via* enteral feeding. Vitamin B group supplementation is also mandatory, especially in patients with alcohol-related cirrhosis[24].

Patients with liver disease undergoing surgery might be at risk of both thrombosis and bleeding, due to dysregulated coagulation and a diminished hemostatic reserve [25-27]. Therefore, cirrhotic patients need to be carefully assessed for the risk of bleeding before surgery. Prothrombin time, international normalized ratio (INR), and platelet count have been formally recommended for the purpose of determining coagulation status in clinical practice. However, the relationship between prothrombin time/INR and the risk of bleeding in cirrhosis is disputable. Isolated evaluations of bleeding or clotting time are also of little prognostic value in patients with liver diseases during pre-operative screening[28].

Fresh frozen plasma and vitamin K therapy (phytomenadione 10 mg/mL) are currently used to address coagulopathy as a standard of care to manage active bleeding or prophylaxis before invasive procedures[29,30]. In cases of urgent surgery, prothrombin complex concentrate (PCC) might be used to prevent bleeding in selected patients with severe coagulopathy. Drebes *et al*[26] recently reviewed PCC's clinical use in patients with acute/chronic liver disease in a retrospective single-center study [26]. In their study, 20–25 IU/kg of PCC was administered to patients with INR 4, and 30 IU/kg for patients with INR 4. PCC therapy effectively improved the results of the coagulation test, with no evidence of an increased risk of thromboembolism. These findings highlighted the need to assess further PCC's potential role as a form of hemostatic therapy in liver disease patients. The use of PCC before urgent surgery should therefore be considered.

Thrombocytopenia is another issue associated with liver cirrhosis. Prophylactical or periprocedural platelet transfusion, splenic artery embolization, transjugular intrahepatic portosystemic shunts, and a splenectomy are well documented methods of treating thrombocytopenia. Recently, thrombopoietin receptor agonists (avatrombopag and lusutrombopag) were approved by the United States Food and Drug Administration for the non-invasive treatment of thrombocytopenia in patients with chronic liver disease undergoing a surgical procedure. These new drugs are considered safe and effective alternatives to platelet transfusions[31,32].

Surgical approach: The importance of the surgical treatment of the UH rupture in patients with ascites was first described by Kirkpatrick and Schubert, who calculated that the mortality rate was 60% in conservatively treated patients, compared to 14% in those who underwent herniorrhaphy[15]. However, there is a much higher risk of complications and a higher morbidity rate after the urgent surgical repair of an UH[33,34].

In terms of the surgical method, most authors suggest that the best option seems to be a primary closure with non-absorbable sutures[5,9,14]. Synthetic meshes may not be an appropriate technique in patients with ascites due to a higher risk of infection and a

possible ingrowth of the mesh[5,35]. However, a single randomized study of 80 patients demonstrated that the use of a synthetic mesh in cirrhotic patients with complicated UHs was related to a lower rate of hernia reoccurrence[16]. UHs reoccurred in 14.2% of patients who underwent suture repair, compared to 2.7% in the mesh repair group ($P < 0.05$). The incidence of infection at the surgical site was not significant in either group: 8.5% in the conventional fascial repair group and 16.2% in the mesh repair group ($P > 0.05$). However, the mean duration of hospital stay was significantly longer in the mesh repair group ($P < 0.05$)[16].

Finally, it is essential to treat these patients in a tertiary center with specialized surgical and intensive care units due to the increased risk of infection, bleeding, and postoperative decompensation of cirrhosis[36]. Smaller hospitals cannot provide appropriate care because of their lack of experience[7,37]. In such cases, several alternative methods are described, including the successful placement of a pig-tail drain with no recurrence of ascitic fluid leakage and the injection of fibrin glue into the defect to stem drainage[38-40]. These approaches allow natural wound healing, though they remain temporary solutions. Ulceration or necrosis over a UH should be considered a dangerous sign; a warning of an impending rupture, and such patients should be referred to urgent surgical treatment[11].

HOW CAN WE ESTABLISH THE INDIVIDUAL RISK FOR HERNIATION AND POSSIBLE HERNIA RUPTURE IN CIRRHOTIC PATIENTS?

The main etiological factors for the development of UHs are ascites, weakened abdominal wall muscles, malnutrition, and the recanalization of pre-existing openings promoted by increased abdominal pressure[5,6,37].

A UH rupture is usually the result of multiple risk factors. Alcohol consumption is one of the major causes of decompensated cirrhosis with ascites. Cirrhosis alone causes malnutrition and sarcopenia, resulting in weakened abdominal wall muscles. Harmful alcohol consumption is also associated with nutritional deficiencies, including protein-calorie malnutrition and cachexia[24,41]. It is also necessary to consider comorbidities that can put limitations on the patient's physical activity and nutrition, such as epilepsy, depression, and related medication (antiepileptics, antidepressants). Previous abdominal surgeries and pre-existing hernias are also severe risk factors for UHs due to the presence of a weakened abdominal wall. Therefore, careful anamnesis is essential (Table 1).

HOW CAN WE PREVENT UH RUPTURES? HOW CAN WE AVOID UH REOCCURRENCE?

General measures for UH prevention consist of the continuous education of the patient and their family members, lifestyle modification, and the avoidance of alcohol, tobacco, constipation, and heavy lifting. Hernia trusses are controversial as they may worsen the hernia, and they should only be used temporarily, if at all[42]

The main methods of preventing UH occurrence and rupture specifically for cirrhotic patients are the optimal control of ascites using medical therapies or regular paracentesis as *per* general guidelines (Table 2)[2,5,17].

The management of underlying liver disease improves the outlook for the reversible components of hepatic decompensation. In most patients with alcoholic cirrhosis, abstinence may reduce or even normalize portal pressure, resulting in the increased ease of treatment of ascites or their disappearance altogether[1]. Alcohol-induced liver cirrhosis is a double pathology, hence the management of both liver cirrhosis and dependence is essential. The main treatment methods are total abstinence, correction of malnutrition, vitamins, and microelements, and the primary and secondary prophylaxis of the complications of cirrhosis. Specific treatment with S–adenosyl–L–methionine, propyluracil, colchicine, and anabolic steroids is debated [41].

It is also essential to monitor carefully the patient with ascites and to consider elective UH repair as soon as herniation appears. This can help to prevent UH rupture and minimize the number of postoperative complications. The watch-and-wait policy was previously applied to patients with ascites and UHs, and surgery was performed only after the occurrence of complications[6]. However, in recent decades, some changes have occurred as novel studies have demonstrated that elective UH repair in

Table 2 Summary of the guidelines on the management of ascites

	Treatment
Main measures	Moderate restriction of sodium intake, 80–120 mmoL/d (4.6-6.9 g of salt/d)
	Adequate nutrition: Protein-rich diet (2000 kcal/d, protein–40-50 g/d), vitamin therapy
	Correction of electrolyte imbalance
	Adequate fluid intake: No restriction needed in patients with normal serum sodium concentration; in hyponatremic patients (< 130 mmoL/L), restrict fluid intake to 1.0-1.5 L/d
	Daily track of weight (or measure fluid intake and diuresis)
	The maximum recommended weight loss during diuretic therapy: (1) 0.5 kg/d in patients without edema; and (2) 1 kg/d in patients with edema
Mild and moderate ascites (grade I°-II°)	Aldosterone antagonists: Spironolactone 50-100 mg/d (maximum of 400 mg/d) ± loop diuretics: Furosemide 20-40 mg/d (maximum of 160 mg/d)
	Torasemide (10-40 mg/d) if no response to furosemide
	Distal diuretics: Amiloride 5-20 mg/d; triamterene 100 mg 2 k./d. (if aldosterone antagonists are not tolerated)
	Combined dosage of diuretics: Spironolactone 50-100-200-300-400 mg/d (in 100 mg steps) + furosemide 20-40-80-120-160 mg/d (in 40 mg steps) (or adequate doses of other diuretics)
Large ascites (grade III°)	LVP
	Albumin infusion (8 g/L of ascitic fluid removed)
	Minimal effective dose of diuretics to prevent the re-accumulation of ascites after LVP
Refractory ascites	Repeated partial or large volume paracentesis + albumin infusion
	Withdrawn diuretics
	Transjugular intrahepatic portosystemic shunt
	Alternative drugs: (1) α1 adrenergic agonists–midodrine 7.5 mg 3 times/d; (2) Vasopressin analog–terlipressin 1-2 mg/d intravenous; and (3) α2 adrenergic agonists–clonidine
	Alfapump system
	Liver transplantation

LVP: Large volume paracentesis.

patients with liver cirrhosis and ascites can be performed with less morbidity. Conservative management leads to a higher incidence of incarceration, with subsequent hernia repair in the emergency setting[34,43,44]. Marsman *et al*[43] compared the outcome of operative and conservative treatment in patients with liver cirrhosis and ascites complicated by UHs. Elective hernia repair was successful without complications or recurrence in around 70.5% of patients, while complications such as wound-related problems (superficial wound infection, necrosis, hematoma) were noted in 17.6% of cases. Initial conservative management was effective in only 23% of patients [41]. A single-center prospective study on patients listed for liver transplantation showed that elective UH repair is preferable to urgent repair in patients with cirrhosis and ascites[44].

Therefore, elective herniorrhaphy is now recommended in patients with cirrhosis and ascites after the patient is stabilized using optimal medical management, to prevent an emergent presentation of incarceration or UH rupture[17,33]. There are several contraindications for elective surgery in patients with liver disease, such as acute liver failure, acute renal failure, acute viral hepatitis, alcoholic hepatitis, cardiomyopathy, hypoxemia, severe coagulopathy, and thrombocytopenia (despite treatment)[17].

Efforts should be taken to avoid recurrent ascites in the postoperative period, as they lead to impaired wound healing and possible risks of dehiscence[17]. Measures for preventing the reoccurrence of the UH after surgery are the same as preventive methods against UH rupture (Table 1).

According to the literature, just 33% of patients with ascites receive only treatments that are supported by guidelines and high-quality evidence[43]. Furthermore, over one-third of patients hospitalized due to cirrhosis complications are readmitted within 1 mo of discharge[45,46]. Many patients do not seek proper care until decompensation,

and some do not follow or cannot afford treatment. Clearly, the current management of cirrhosis leaves room for improvement, especially during the conditions of the coronavirus disease 2019 (COVID-19) pandemic.

HOW CAN WE MANAGE PATIENTS IN THE CONDITIONS CREATED BY THE COVID-19 PANDEMIC?

Clinicians must be aware that the COVID-19 pandemic has negatively impacted the care of patients with chronic liver disease[47]. This might result in a higher incidence of decompensated cirrhosis when patients do not receive treatment correction on time. There is also an increased risk of a growing incidence of alcohol-induced and non-alcoholic fatty liver disease-related cirrhosis. Patient care is challenging; along with telemedicine and the near-impossibility of physical examination, clinicians must be more accurate and foresee potential future issues. Moreover, not every patient can or indeed wants to use telemedicine, as many patients represent vulnerable cohorts. However, there are some steps that can be taken to address these challenges.

First, we must make more effort than ever before to encourage and educate patients and their relatives. Visual methods can be used, such as written instructions for the patient (in a patient-friendly form), educational video seminars, and other materials. We must ask key questions (during teleconsultation) to establish the patient's risks (Table 1), and we must cooperate. Cooperation between patient and doctor and communication between different medical specialists is essential in managing patients with liver cirrhosis and ascites. All clinicians must be aware of the possible complications of ascites and refer the patient to a specialist consultation in a timely manner.

CONCLUSION

The rupture of the UH and spontaneous paracentesis in patients with decompensated cirrhosis is a rare and dangerous complication that remains a challenge for gastroenterologists, surgeons, and anesthesiologists. The stabilization of the patient's overall condition, adequate control of ascites, and local wound care are the main goals of therapy before the primary repair of the hernia. Gastroenterologists and general practitioners should establish the risk to the individual patient regarding herniation and possible hernia rupture *via* careful follow-up. Timely prevention of UH and elective surgical treatment can reduce the risk of complications and the need for urgent surgery, decrease overall hospitalization time, and improve the patient's quality of life.

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REVIEW

Radiomics and machine learning applications in rectal cancer: Current update and future perspectives

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Abstract

The high incidence of rectal cancer in both sexes makes it one of the most common tumors, with significant morbidity and mortality rates. To define the best treatment option and optimize patient outcome, several rectal cancer biological variables must be evaluated. Currently, medical imaging plays a crucial role in the characterization of this disease, and it often requires a multimodal approach. Magnetic resonance imaging is the first-choice imaging modality for local staging and restaging and can be used to detect high-risk prognostic factors. Computed tomography is widely adopted for the detection of distant metastases. However, conventional imaging has recognized limitations, and many rectal cancer characteristics remain assessable only after surgery and histopathology evaluation. There is a growing interest in artificial intelligence applications in medicine, and imaging is by no means an exception. The introduction of radiomics, which allows the extraction of quantitative features that reflect tumor heterogeneity, allows the mining of data in medical images and paved the way for the identification of potential new imaging biomarkers. To manage such a huge amount of data, the use of machine learning algorithms has been proposed. Indeed, without prior explicit programming, they can be employed to build prediction models to support clinical decision making. In this review, current applications and future perspectives of artificial intelligence in medical imaging of rectal cancer are presented, with an imaging modality-based approach and a keen eye on unsolved issues. The results are promising, but the road ahead for translation in clinical practice is rather long.

Key Words: Rectal cancer; Radiomics; Radiogenomics; Artificial intelligence; Machine

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Core Tip: Rectal cancer is a common malignancy requiring a multidisciplinary approach to ensure the best clinical management. Diagnostic imaging has contributed to increased survival rates and provided crucial information on the course of rectal cancer patients. Artificial intelligence, and in particular radiomics and machine learning, are promising techniques that could further enhance the value of medical imaging, allowing the building of decision support tools based on quantitative data. We herein present and discuss the potential role of artificial intelligence in rectal cancer applied to different medical imaging modalities.

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INTRODUCTION

In 2020, more than 40,000 cases of rectal cancer (RC) were expected in the United States alone, with a higher incidence in men than in women and a median age at diagnosis of 63 years[1]. However, over the past years there has been an improvement in RC management associated with a reduction of mortality and higher survival rates, mainly related to earlier diagnosis and more effective treatment[2]. While endoscopy represents the gold standard for RC diagnosis, there are several factors to be considered that influence prognosis and therapeutic strategy, including local tumor extent (T), lymph nodes status (N) and presence of distant metastases (M)[3]. Indeed, radical surgery with curative intent (*i.e.* total mesorectal excision, TME) is recommended as a first-line strategy in patients with locally confined disease after neoadjuvant chemoradiotherapy (nCRT) for locally advanced RC (LARC). Metastatic patients, on the other hand, usually undergo systemic therapies such as chemotherapy, targeted therapy, or immunotherapy[4,5]. Diagnostic imaging plays a crucial role for pretreatment disease staging, with a multimodal approach commonly being necessary [6]. Magnetic resonance imaging (MRI) is regarded as the most valuable imaging modality for primary loco-regional staging of RC and restaging after nCRT[7,8]. Computed tomography (CT) scans are routinely performed to detect distant metastases, with the most common metastatic sites being the liver and lungs[2]. Currently, hybrid imaging by positron emission tomography/CT (PET/CT) could provide useful prognostic data for RC, even if its role still remains to be defined[6,9]. Likewise, the potential of simultaneously acquired PET and MRI still has to be explored[10]. However, conventional image assessment has recognized limitations that are driving the research towards the identification and validation of novel strategies to further increase the value of diagnostic imaging[11-13]. In this setting, a post processing quantitative technique known as radiomics appears particularly promising, with encouraging evidence collected in recent years[14,15]. Radiomics has been frequently and successfully coupled with artificial intelligence (AI), and in particular machine learning (ML) approaches in the field of oncologic imaging[16-19]. This review aims to introduce readers to the concepts of radiomics and ML and to present the state-of-the-art of RC radiomics-ML applications, with an imaging modality-based approach, highlighting their strengths and drawbacks.

RADIOMICS AND ML: WHAT, WHY, AND HOW

Trying to quantify what is visually assessed in medical imaging is a rather difficult task, and radiologists have traditionally provided qualitative information and semi-quantitative data in their reports[20]. However, this leads to a large amount of unused

data remaining hidden in medical images[21]. Furthermore, semantic descriptors of cancer imaging phenotype (*e.g.*, "central necrosis", "irregular margin", and "diffusely heterogeneous") are prone to poor intra- and interobserver reliability, experience dependent, and might not significantly reflect actual tumor biology[22]. Indeed, tumors are not considered to be homogeneous entities but instead composed of various cell clones with biologically relevant differences[23]. Radiomics allows the conversion of images into mineable data with the high-throughput extraction of quantitative parameters (*i.e.* radiomics features) that capture the heterogeneities and provide important information on cancer phenotype[24]. Radiomics is a multistep process beginning with image acquisition and followed by image segmentation, which is the two- or three-dimensional delineation of the region of interest (ROI), usually represented by the primary tumor. Image segmentation can be manual, performed by a human operator; semiautomatic, performed by AI and manually adjusted; or automatic, exclusively performed by AI[25] . Subsequently, hundreds of radiomics features can be extracted from the ROI using specifically designed formulae conveying different information, including shape, first-order (based on the distribution of pixel intensities), second and higher-order features (accounting for pixel intensities spatial distribution)[26]. Correlating radiomics features to the outcomes of interest is the endpoint of radiomics, and many believe it could open the gateway to precision medicine[27,28]. However, such a huge amount of data can be more easily handled by AI rather than traditional statistical methods[21]. Indeed, ML is a branch of AI focused on algorithms that can be trained for a task they were not specifically programmed to perform[29]. The algorithms are essentially used for classification problems, with the main oncologic imaging application being decision support in various settings that include detection, characterization, and monitoring[30-32]. To properly train an ML algorithm, "the curse of dimensionality," which is a set of issues arising when working with a number of features much higher than the patient population must be avoided. Feature reduction can be achieved in several ways that may also be combined to achieve better results[33,34]. Indeed, an excessive number of features increases the chances of finding nongeneralizable correlations (*i.e.* overfitting). On the other hand, complex relationships might need more features to build a proper prediction model [35]. Finally, trained ML classifiers need to be tested to verify generalizability on external data not used in the training process and possibly provided by different institutions[36]. A kind of ML algorithm called deep learning (DL), based on neural networks (NN), does not necessarily require image segmentation and learns autonomously the best features for performing data classification[37]. A brief description of the most commonly applied ML algorithms in RC radiomics can be found in Table 1.

RADIOMICS AND ML APPLICATIONS IN RC: MRI

Thanks to its superb contrast resolution, MRI plays a pivotal role in the diagnostic pathway of RC patients, particularly for primary local staging and restaging after treatment[38]. Indeed, in addition to T and N staging, MRI provides valuable information such as the circumferential resection margin, defined as the minimum distance between the tumor and the mesorectal fascia, as well as extramural venous invasion (EMVI), an independent negative prognostic factor for RC[39,40]. In the following paragraphs, radiomics and ML approaches proposed to further increase the value of MRI in the assessment of RC are described.

Staging

Currently, MRI represents the first-choice imaging modality for determining RC local extent. However, the assessment of T stage is a challenging task, and staging failures often occur in the differentiation between T2 in which the tumor involves the muscularis propria and T3, in which the tumor involves perirectal tissue beyond the muscularis propria[41]. Decision support tools based on MRI radiomics and ML might be able to aid radiologists in this endeavor[42-44]. Using multilayer perceptron, a DL model powered by T2-weighted (T2w) radiomics features from pretreatment MRI, Ma *et al*[42] were able to discriminate between patients with T1 or T2 and those with T3 or T4 RC with 76% sensitivity and 74% specificity. Similar results were found using diffusion-weighted imaging (DWI) to extract radiomics features in a recent investigation on 115 patients. A logistic regression (LR) algorithm reached a sensitivity of 79% and a specificity of 74% for the same classification problem[43]. Finally, an LR model built with T2w images, both with and without fa-suppression, radiomics

Table 1 Overview of the most widely adopted machine learning algorithms in rectal cancer imaging

Algorithm name	Description
Random forest	An ensemble method that combines multiple decision trees (a class of predictive learning models used in supervised ML) to obtain more accurate results for classification and regression tasks
Support vector machine	A linear approach used mainly for classification problems with the aim to find the best hyper plane which most accurately separate input data into two classes
Logistic regression	A classifier used to obtain the best fitting model for the relationship between multiple predictor variables and a dichotomous outcome
LASSO	A regularized regression method that performs both variable selection and regularization in order to optimally fit the resulting generalized statistical model
Naive Bayes	A classifier relying on the Bayes Theorem to model the probability of an outcome based on the strong (naive) independence assumptions between the features data
Quadratic discriminant analysis	A subtype of Dimensionality Reduction Algorithms that turn high-dimensional data into to low-dimensional data retaining the most significant features of original data for the prediction of the class label
ANN	A subgroup of ML composed of neuronal-like multi-layered networks allowing to automatically extract features without prior labelling and perform complex operations
CNN	As subset of ANN containing multiple computational hidden layers that filter and compute high-dimensional data to enhance the learning of high-level tasks (deep learning)

ANN: Artificial neural network; CNN: Convolutional neural network; LASSO: Least absolute shrinkage and selection operator; ML: Machine learning.

features achieved a sensitivity of 88% and specificity of 61% for classifying T1-2 *vs* T3-4 in a group of 174 patients[44].

MRI is also considered the imaging gold standard for the assessment of lymph node involvement in RC, but it suffers from a relatively low specificity, with potential negative implications on patient outcome[45]. Indeed, the management of patients with different nodal status is a highly debated and complex topic[46]. Radiomics has been proposed as a feasible solution to enhance the accuracy of MRI for N staging in RC patients[47]. In a recent retrospective single-center study in 152 patients, T2w radiomics were coupled to a random forest (RF) algorithm to create an ML classifier that was able to discriminate N0 from N1-2 patients with a sensitivity of 79% and a specificity of 72%[42]. Once again, similar results (81% sensitivity and 68% specificity) were found with LR and a different ML model derived from DWI radiomics features [43]. In both studies, pretreatment MRI scans were used, and the primary tumor was segmented. With a different approach, Zhu *et al*[48] extracted collective radiomics features from all noticeable lymph nodes on T2w images acquired before and after nCRT in patients with LARC; the LR model was trained to predict pathological node status after nCRT with a group of 143 patients, and had a sensitivity of 95% and a specificity of 60% in the validation cohort of 72 patients. The sensitivity was slightly lower and the specificity slightly higher than those reached by radiologist in the same patient cohorts (100% and 43%). Notwithstanding the specificity insufficient for clinical needs, such models might be useful tools for radiologists in the assessment of N stage in RC.

Finally, the identification of distant metastases in RC patients usually relies on imaging modalities other than MRI. Nevertheless, it should be mentioned that radiomics of the primary tumor was able to provide valuable information for the prediction of synchronous (already present at the time of diagnosis) or metachronous (developed after treatment) liver metastases[49-51] as well as synchronous metastases to other sites[52]. With specific regard to metachronous liver metastases, radiomics of T2w and post-contrast T1-weighted dynamic contrast enhanced (DCE) images were combined to build two ML predictive models, a support vector machine (SVM) and LR, with cross-validation in 108 patients[50]. The LR algorithm had the best performance, but not significantly better than SVM, with 83% sensitivity and 76% specificity, confirming the potential of radiomics and ML for the identification of RC patients who will develop liver metastases after treatment.

Predicting response to nCRT in patients with LARC

While TME should follow nCRT in patients with LARC, the role of surgery in patients with a complete response to nCRT is currently debated, and a "watch and wait" strategy has been proposed[53]. Indeed, patients who achieve a pathological complete response (pCR) after nCRT have better long-term outcomes compared with non-pCR

patients, and could therefore be managed differently[54]. Unfortunately, pCR cannot be accurately predicted before surgery by conventional evaluation of MR images[55]. Recently, several radiomics features extracted from T2w, DWI, and DCE sequences have been investigated as possible imaging biomarkers for pCR prediction, with promising results[56-58]. The main studies that aimed to build classification models using ML algorithms for preoperative prediction of pCR after nCRT are shown in Table 2. Overall, the performance of the different models is encouraging. While a trend can be observed, with lower values found in those studies that validated the model in an external dataset and thus with the better chances of high generalizability, it is difficult to draw a final conclusion from the available evidence[59,60]. Most studies focused on MRI scans acquired before nCRT had started, extracting radiomics features from highly available sequences (*i.e.* T2w). The ideal approach exploits an advantage of radiomics that allows developing predictive models using medical images as they are acquired in the clinical routine[61]. On the other hand, each of the retrospective studies presented its own model, with a certain degree of heterogeneity that does not facilitate translation into clinical practice. An overview of the main studies proposing MRI radiomics and ML algorithms for the prediction of nCRT outcomes other than pCR is reported in Table 3. In those studies, the ML models were generally designed to classify patients into two groups (*i.e.* good and poor responders to nCRT), with one study prospectively designed but lacking external validation[62].

Additionally, recent studies explored the feasibility of radiomics nomograms, based on the combination of a radiomics signature and either a pretreatment MRI T stage[63] or a post treatment tumor length[64], to predict pCR to nCRT. In particular, Liu *et al* [64] built and validated a radiomics signature in LARC patients using T2w in 152 and DWI images in 70 the T2w and DWI images were acquired both before and after nCRT. An SVM ML algorithm incorporating signatures and post treatment tumor length in a nomogram was able to reach a final diagnostic accuracy of 94% in the prediction of pCR. Finally, Wang *et al*[65] developed a radiomics signature to classify good responders and poor responders to nCRT with an LR ML algorithm and radiomics features from T2w, DWI, and DCE sequences. When combined in a nomogram with MRI T stage and circumferential resection margin as well as apparent diffusion coefficient values, they were able to predict a good response with a sensitivity of 71% and a specificity of 88%.

Genotyping

Radiogenomics aims to correlate imaging features of a disease with its genotypic characteristics and represents the next step in a radiology-pathology correlation[66]. Radiomics and radiogenomics are not equivalent, and both qualitative and quantitative imaging features can be used for radiogenomic analysis, with quantitative data having promising associations with genetic mutations in RC[67]. Among the negative genetic prognostic factors in RC, KRAS mutations are associated with poor response to epidermal growth factor receptor-targeted antibodies[68] and an increased risk of developing distant metastases[69]. In a recent multicenter study by Cui *et al* [70], three classifiers (decision tree, SVM and LR) powered by T2w-based radiomics features were trained to predict KRAS mutations in data from 213 patients and validated in both an internal cohort of 91 patients and external cohort of 86. The SVM obtained the greatest area under the receiver operating characteristic curve (AUC) in the training dataset (0.72), which was substantially confirmed in the internal (AUC = 0.68) as well as external (AUC = 0.71) validation cohorts. The finding supports the potential generalizability of such models. Interestingly, in the same study, no associations were found between KRAS status and baseline clinical and histopathological data. More optimistic results were recently published using a decision tree classifier (AUC = 0.88) by a different study group[71], but the sample size was substantially smaller (60 patients) and the model was not externally validated. Finally, T2w-based radiomics have been also paired with DL, with an artificial NN discriminating between patients with or without KRAS mutations and a classification error of 13% [72].

Assessing high-risk histopathological variables

Several histopathological characteristics, EMVI, differentiation degree, and perineural invasion (PNI) for example, are associated with poor clinical outcome and need to be considered in the risk stratification of patients with RC. It is fair to assume that a reliable pretreatment evaluation of these high-risk variables would ease the transition toward precision medicine[5]. ML classifiers applied to MRI radiomics features have been recently explored in this setting[73-75]. As previously highlighted, MRI can be used to identify EMVI; however, its sensitivity is not as high as desirable[76]. To

Table 2 Key characteristics of the main studies using radiomics and machine learning algorithms on magnetic resonance images to predict pathologic complete response after neoadjuvant chemoradiotherapy in patients with locally advanced rectal cancer

Ref.	Study design (*n* of sites)	Number of patients	Definition of pCR	MRI field strength (*n* of scanners)	MRI timing	MRI sequence	ML algorithm	Data powering algorithm	Validation	Performance (AUC)
Antunes *et al* [59], 2020	Retrospective (3)	104	TRG 0 according to AJCC	1.5 and 3 T (> 10)	Pre-nCRT	T2w	RF	Radiomics features	External validation	0.71
Ferrari *et al* [106], 2019	Retrospective (1)	55	TRG 4 according to Dowrak-Rodel	3 T (1)	Pre-, mid- and post-nCRT	T2w	RF	Radiomics features	Internal validation (train/test split)	0.86
Horvat *et al* [107], 2018	Retrospective (1[1])	114	ypT0N0	1,5 and 3 T (4)	Post-nCRT	T2w	RF	Radiomics features	Internal validation (cross-validation)	0.93
Nie *et al* [108], 2016	Retrospective (1)	48	ypT0N0	3 T (1)	Pre-nCRT	T2w, DWI, pre and post-contrast T1w	ANN	Radiomics features	Internal validation (cross-validation)	0.84
Petkovska *et al*[109], 2020	Retrospective (1[1])	102[2]	ypT0N0	1,5 and 3 T (4)	Pre-nCRT	T2w	SVM	Radiomics and semantic features	Internal validation (train/test split)	0.75
Shaish *et al* [110], 2020	Retrospective (2)	132	ypT0N0	1,5 and 3 T (multiple[3])	Pre-nCRT	T2w	LR	Radiomics features	Internal validation (train/test split)	0.80
Shi *et al* [111], 2019	Retrospective (1)	51	TRG 0 according to Ryan	3 T (1)	Pre- and mid-Ncrt[4]	T2w, DWI, pre- and post-contrast T1w	CNN	Radiomics features	Internal validation (cross-validation)	0.83
van Griethuysen *et al*[60], 2019	Retrospective (2)	133	ypT0/TRG1 according to Mandard	1,5 T (3)	Pre-nCRT	T2w and DWI	LR	Radiomics features	External validation	0.77
Yi *et al*[112], 2019	Retrospective (1)	134	ypT0N0	1,5 and 3 T (2)	Pre-nCRT	T2w	SVM	Radiomics, clinical and semantic features	Internal validation (train/test split)	0.88

[1]< 10% of scans from other institutions.

[2]All previously included in Horvat *et al*[107], 2018.

[3]Inclusion of patients with MRI performed elsewhere but treated at study sites.

[4]Both MRI scans were not available for all patients. In all studies, three-dimensional manual segmentation of the primary tumor was performed to extract radiomic features, except for Shaish *et al*[110], mesorectal compartment) and van Griethuysen *et al*[60] (semiautomatic segmentation). ANN: Artificial neural network; CNN: Convolutional neural network; AUC: Area under the receiver operating characteristic curve; DWI: Diffusion-weighted imaging; LR: Logistic regression; ML: Machine learning; MRI: Magnetic resonance imaging; nCRT: Neoadjuvant chemoradiotherapy; RF: Random forest; SVM: Support vector machine; T1w: T1-weighted; T2w: T2-weighted; TRG: Tumor regression grade.

overcome current MRI limitations, Yu *et al*[73] built a nomogram based on both a DCE MRI radiomics signature and clinical data, finding that it outperformed conventional quantitative perfusion parameters such as Ktrans in the prediction of EMVI, with a sensitivity of 88.9% and a specificity of 78.3% in the validation cohort.

Well-differentiated tumors are associated with better outcomes of RC patients[77]. In a large cohort of 345 patients retrospectively enrolled at a single institution, Meng *et al*[74] explored the performance of three ML classifiers, RF, SVM, and least absolute shrinkage, and selection operator (LASSO) to identify well-differentiated RC. Radiomics features were extracted from multiple MRI sequences, including T2w, DWI, and DCE. The LASSO algorithm had the best performance, with an AUC of 0.72 in the validation dataset. Finally, PNI, the tumor spreading along the nerve sheath, is a histopathological factor known to be associated with poor prognosis[78]. Using T2w

Table 3 Key characteristics of the main studies using radiomics and machine learning algorithms on magnetic resonance images to predict outcome other than pathologic complete response after neoadjuvant chemoradiotherapy in patients with locally advanced rectal cancer

Ref.	Study design (*n* of sites)	Number of patients	Prediction task	CT phase (*n* of CT scanner)	Segmentation method	ML algorithm	Data powering algorithm	Validation	Performance
Bibault *et al*[85], 2018	Retrospective (3)	99	pCR after nCRT	Unenhanced (3)	Manual – 3D	DNN	Radiomics and clinical features	Internal validation (cross-validation)	AUC: 0.72
Hamerla *et al*[86], 2019	Retrospective (1)	169	pCR after nCRT	Unenhanced (1)	Manual – 3D	RF	Radiomics features	Internal validation (cross-validation)	Accuracy: 0.87
Yuan *et al* [87], 2020	Retrospective (1)	91	pCR after nCRT	Unenhanced (1)	Manual – 3D	RF	Radiomics features	Internal validation (train/validation split)	Accuracy: 0.84
Wu *et al* [90], 2019	Retrospective (1)	102	MSI status	Venous phase - DECT (2)	Manual - 3 2D ROIs for lesion	LR	Radiomics features	Internal validation (train/validation /test split)	AUC: 0.87
Fan *et al* [91], 2019	Retrospective (1)	100	MSI status	Portal venous phase (2)	Semiautomatic – 3D	NB	Radiomics features	Internal validation (cross-validation)	AUC: 0.75
Wu *et al* [92], 2020	Retrospective (1)	173	KRAS mutation	Portal venous phase (3)	Manual + DL – single 2D ROI	LR	Radiomics features	Internal validation (train/test split)	C-index: 0.83
Wang *et al*[94], 2019	Retrospective (1)	411	Prediction of survival	Unenhanced (1)	Manual – 3D	10-F CV	Radiomics and clinical features	Internal validation (cross-validation)	C-index: 0.73

In all studies, three-dimensional manual segmentation of the primary tumor was performed to extract radiomic features, with the exceptions of Alvarez-Jimenez *et al*[113] (rectal wall), van Griethuysen *et al*[60] (semiautomatic segmentation) and Yang *et al*[115] (two-dimensional manual segmentation). ANN: Artificial neural network; AUC: Area under the receiver operating characteristic curve; CNN: Convolutional neural network; DWI: Diffusion-weighted imaging; EMLM: Ensemble machine learning model; GR: Good responders; LASSO: Least absolute shrinkage and selection operator; RF: Random forest; LR: Logistic regression; ML: Machine learning; MRI: Magnetic resonance imaging; QDA: Quadratic discriminant analysis; SVM: Support vector machine; T1w: T1-weighted; T2w: T2-weighted; TRG: Tumor regression grade.

10F-CV: 10-fold cross-validation; CT: Computed tomography; DECT: Dual-energy computed tomography; DNN: Deep neural network; LR: Logistic regression; ML: Machine learning; MSI: Microsatellite instability; NB: Naive Bayes; nCRT: Neoadjuvant chemoradiotherapy; pCR: Pathologic complete response; RF: Random forest.

radiomics and AI, Chen *et al*[79] developed a nomogram to predict the presence of PNI in RC patients (AUC = 0.85). A decision curve analysis confirmed the clinical utility of their nomogram, but the sample size of only seven PNI-positive patients in the test dataset requires validation of this preliminary findings in larger datasets.

RADIOMICS AND ML APPLICATIONS IN RC: CT

In the management of RC, CT is commonly used as the initial staging modality, allowing accurate nodal and metastases staging and target volume delineation before radiation therapy in patients with LARC[6]. Conversely, the role of CT in RC pretreatment local staging as well as restaging after nCRT is limited because of its intrinsically lower contrast resolution compared with MRI[80,81]. Nevertheless, much effort has been directed toward the use of CT data beyond clinical indications, with the aim of developing CT-based radiomics signatures reflecting tumor heterogeneity[82]. CT images contain robust volumetric data that are highly reproducible across patients and are an ideal source of data to feed AI systems[83,84]. In that perspective, ML models that can find correlations of RC CT radiomics features that can be used to predict outcomes such as complete response to nCRT in LARC patients, genetic profiles, overall survival, and segmentation (Table 4).

Table 4 Key characteristics of the main studies using radiomics and machine learning algorithms on computed tomography for v prediction tasks

Ref.	Study design (*n* of sites)	Number of patients	Prediction task	CT phase (*n* of CT scanner)	Segmentation method	ML algorithm	Data powering algorithm	Validation	Performance
Bibault *et al*[85], 2018	Retrospective (3)	99	pCR after nCRT	Unenhanced (3)	Manual – 3D	DNN	Radiomics and clinical features	Internal validation (cross-validation)	AUC: 0.72
Hamerla *et al*[86], 2019	Retrospective (1)	169	pCR after nCRT	Unenhanced (1)	Manual – 3D	RF	Radiomics features	Internal validation (cross-validation)	Accuracy: 0.87
Yuan *et al* [87], 2020	Retrospective (1)	91	pCR after nCRT	Unenhanced (1)	Manual – 3D	RF	Radiomics features	Internal validation (train/validation split)	Accuracy: 0.84
Wu *et al* [90], 2019	Retrospective (1)	102	MSI status	Venous phase - DECT (2)	Manual - 3 2D ROIs for lesion	LR	Radiomics features	Internal validation (train/validation /test split)	AUC: 0.87
Fan *et al* [91], 2019	Retrospective (1)	100	MSI status	Portal venous phase (2)	Semiautomatic – 3D	NB	Radiomics features	Internal validation (cross-validation)	AUC: 0.75
Wu *et al* [92], 2020	Retrospective (1)	173	KRAS mutation	Portal venous phase (3)	Manual + DL – single 2D ROI	LR	Radiomics features	Internal validation (train/test split)	C-index: 0.83
Wang *et al*[94], 2019	Retrospective (1)	411	Prediction of survival	Unenhanced (1)	Manual – 3D	10-F CV	Radiomics and clinical features	Internal validation (cross-validation)	C-index: 0.73

10F-CV: 10-fold cross-validation; CT: Computed tomography; DECT: Dual-energy computed tomography; DNN: Deep neural network; LR: Logistic regression; ML: Machine learning; MSI: Microsatellite instability; NB: Naive Bayes; nCRT: Neoadjuvant chemoradiotherapy; pCR: Pathologic complete response; RF: Random forest.

Predicting response to nCRT in patients with LARC

Bibault *et al*[85] explored the reliability of deep NN (DNN) integrating clinical features (T stage) and robust radiomics CT-based features in assessing the pCR to nCRT in a multicenter cohort of patients with LARC. The DNN model predicted pCR with an accuracy of 80% compared with 69.5% achieved with an LR model using only the TNM stage and an SVM model with the same parameters as the DNN that had an accuracy of 71.58%. Similarly, Hamerla *et al*[86] reported an accuracy of 87% for prediction of pCR after nCRT using an ML algorithm and CT radiomics data, but they noted that the model was not generalizable because of bias introduced by an imbalanced distribution of the minority class (pCR: 13% and non-pCR = 87%) in the study population. In another study, Yuan *et al*[87] tested and compared different ML algorithms using robust CT-based radiomics features significantly correlated with pCR. The best performing model was an RF with an accuracy of 83.9% in the test population. Interestingly, these studies used radiomics features extracted from unenhanced CT scans used for radiotherapy planning. The process highlights the potential value of nonroutine CT data for pretreatment risk stratification.

Genotyping

Recent studies have shown encouraging results with regard to the high predictive ability of AI-radiomics CT-based models of the biologic behavior of RC, in terms of microsatellite instability (MSI) status and KRAS gene mutations, which are considered significant molecular markers of improved prognosis and adjuvant therapy[11,88,89]. Wu *et al*[90] developed a pretreatment predictive model of MSI status in RC using ML radiomics features extracted from venous phase images of iodine-based material decomposition with dual-energy CT (DECT). Performance of the model was tested on images acquired with a different DECT scanner, and achieving a diagnostic accuracy of 79%. The result suggests a possible link between iodine DECT images and augmented tumor vascularization. In a preliminary retrospective study, Fan *et al*[91] found that an ML model combining clinical and CT radiomics features had a better classification performance for MSI status (AUC = 0.75) in stage II RC patients than models using only clinical features (AUC = 0.60) or only radiomics features (AUC = 0.70).

Another research group[92] investigated the performance of a model that used handcrafted radiomics signatures combined with those in a DL algorithm. The combined model was able to the discriminate patients with mutant or the wild-type KRAS with a sensitivity of 80% and a specificity of 72% in the validation cohort, thus showing a good predictive performance.

Prognosis

An active field of AI oncology-related research is the discovery of new clinical and imaging tumor biomarkers that are correlated with prognosis, with the goal of developing accurate predictive models of treatment response based on personalized tumor profiles[93]. Wang *et al*[94] explored the use of CT-based ML models powered by clinical and radiomics features to assess the prognostic outcomes of LARC patients treated with nCRT. Radiomics features were extracted from nonenhanced CT images used for planning the treatment of 411 LARC patients. Images analyzed by unsupervised ML did not find a relationship between the clinical and radiomics features. A supervised ML model with embedded radiomics and clinical parameters had an improved overall survival prediction in the testing set and a c-index of 0.73 which was significantly better (P = 0.044) than the performance of the model using only clinical factors (c-index = 0.67).

RADIOMICS AND ML APPLICATIONS IN RC: MULTIMODAL AND HYBRID IMAGING

The advantage of multimodality and hybrid imaging in oncology is mainly related to the combined evaluation of anatomical and functional tumor characteristics. Radiomics and ML could further increase the potential value of the techniques[95]. However, the number of studies evaluating RC is still limited, and the role of multimodal radiomics and ML models has mainly been investigated for the prediction of response to nCRT in patients with LARC[96,97]. In a single-center study in 169 patients, Shen *et al*[96] developed an RF model based on baseline PET/CT images that accurately predicted pCR to nCRT in LARC patients, with a sensitivity of 81.8% and a specificity of 97.3%. Another study confirmed the feasibility of combining pretreatment MRI data from T2w sequences and PET radiomics features to build a prediction model able to identify responders or nonresponders. ML algorithms were used for semiautomatic segmentation of the primary tumor in both sets of images[97]. The final LR model had a sensitivity of 86% and specificity of 83%. Beyond nuclear medicine, Li *et al*[98] described a multimodal radiomics-based nomogram with features extracted from baseline MRI and CT images, which better performed better than individual imaging techniques in the prediction of response to nCRT. Although multimodal radiomics for RC is in its infancy, the encouraging preliminary reports support the idea that it could allow an even more comprehensive assessment of tumor characteristics compared with individual images.

CURRENT LIMITATIONS AND FUTURE PERSPECTIVES

The available evidence confirms that AI is a feasible tool to broaden the spectrum of information that medical imaging can provide for the management of RC patients. Nevertheless, there is a risk that negative results are not published because of publication bias[99]. Furthermore, what could theoretically be done is not ready for clinical practice at present. Indeed, there are many exploratory studies and very few confirmatory ones to support the use of one radiomics-ML model over another. A possible solution to the problems of verifying generalizability and comparing the performance of different models proposed for the same prediction task might be the use of open-source data[100]. Indeed, a publicly available large dataset from multiple institutions could serve as a common benchmark to verify whether the available models can reproduce previous results while we wait for well-designed prospective clinical trials that will overcome the limitations of retrospective studies. Currently, there is a great interest in public imaging datasets, but their quality might be heterogeneous[101]. It should also be considered that significant variations in radiomics and ML pipelines make it difficult to compare studies. Adherence to shared guidelines for AI study design is this highly advisable[102]. Another issue of concern that could prevent widespread adoption of radiomics-ML prediction models is manual

segmentation. It is often necessary, but is a time-consuming procedure that requires automatization. However, AI could also solve that problem. Recent studies have described the use of DL for fully automated segmentation of RC on both CT and MR images, with encouraging accuracy and computational time results[103,104]. Several of the radiomics-ML models described in this review had promising accuracy, but it should be noted that the potential clinical utility of such models depends on multiple factors, such as their added value in comparison with current gold standards, the cost-effectiveness of their implementation, and their actual impact on clinical practice. Decision curve analysis might be helpful in the analysis[34]. Finally, a recent study found that the overall quality of radiomics studies in oncology is below the desired standards, suggesting that most of the problems identified in the field of RC radiomics are shared among the studies involving different types of cancer[105].

CONCLUSION

Medical images contain mineable data with great potential. AI appears to be a convenient tool to harness their value for RC management. AI in imaging can support physicians in the transition toward precision medicine for RC patients, but there is still a long road ahead and it is time to start moving to the next step. Robust prospective multicenter studies and clinical trials are needed to confirm the clinical implications of this new methodology.

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REVIEW

Could the burden of pancreatic cancer originate in childhood?

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Abstract

The presence of pancreatic cancer during childhood is extremely rare, and physicians may be tempted to overlook this diagnosis based on age criteria. However, there are primary malignant pancreatic tumors encountered in pediatric patients, such as pancreatoblastoma, and tumors considered benign in general but may present a malignant potential, such as the solid pseudo-papillary tumor, insulinoma, gastrinoma, and vasoactive intestinal peptide secreting tumor. Their early diagnosis and management are of paramount importance since the survival rates tend to differ for various types of these conditions. Many pediatric cancers may present pancreatic metastases, such as renal cell carcinoma, which may evolve with pancreatic metastatic disease even after two or more decades. Several childhood diseases may create a predisposition for the development of pancreatic cancer during adulthood; hence, there is a need for extensive screening strategies and complex programs to facilitate the transition from pediatric to adult

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healthcare. Nevertheless, genetic studies highlight the fact the specific gene mutations and family aggregations may be correlated with a special predisposition towards pancreatic cancer. This review aims to report the main pancreatic cancers diagnosed during childhood, the most important childhood diseases predisposing to the development of pancreatic malignancies, and the gene mutations associates with pancreatic malignant tumors.

Key Words: Pancreatic cancer; Pancreatic metastasis; Childhood; Adult; Screening; Transition

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Core Tip: Pancreatic malignant tumors are rarely found during childhood, their prognosis being linked to the type of tumor and its capacity to evolve towards metastasis. Also, there are types of cancer diagnosed in pediatric patients which may present with pancreatic metastasis later, during adulthood. Various conditions, when diagnosed during childhood, may be associated with the later onset of pancreatic cancer. Also, several genetic mutations have been linked to the development of pancreatic malignancies. We discuss here the above-mentioned topics in the context of a comprehensive literature review.

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INTRODUCTION

In spite of technological, economic and healthcare progress, pancreatic cancer remains correlated with high mortality rates, its diagnosis at an early stage being of paramount importance for a favorable outcome, but this objective has proven exceedingly difficult to achieve. Researchers in the field have attempted to correlate the appearance of malignant pancreatic tumors with various local or systemic diseases and/or genetic mutations. The preventive management in cases of high risk for pancreatic cancer begins during childhood, by assessing and, whenever possible, treating the predisposing factors[1].

Pancreatic tumors are rarely found among the pediatric population; therefore, their study is limited to isolated case reports or case series. From a histological point of view, pancreatic tumors differ during childhood and adulthood, with a tendency for a better prognosis and clinical outcome at younger ages[2].

This review aims at reporting the primary pancreatic malignant tumors encountered during childhood, the pediatric cancers with the capacity to evolve towards pancreatic metastatic disease, the conditions diagnosable at a pediatric age that may predispose to pancreatic cancer during adulthood, and the most important gene mutations correlated with a predisposition towards pancreatic cancer (Table 1).

PANCREATIC MALIGNANT TUMORS IN CHILDREN

In general, pancreatic tumors are divided into epithelial (exocrine, of acinar, ductal, or undetermined cell origin; or endocrine) and non-epithelial types. The most frequent pancreatic tumor among children is of acinar cell origin, pancreatoblastoma. Solid pseudo-papillary tumors present a low malignant potential and are mostly encountered in young female patients. Rare types of pancreatic cancer may be found in children, such as acinar cell carcinoma and the extremely rare ductal adenocarcinomas. In rare cases, neonatal hypoglycemia can be caused by a focal or diffuse neuroendocrine adenoma of the gland[3].

Table 1 Pediatric pancreatic cancers

Pediatric pancreatic malignant tumors
Pancreatoblastoma
Solid pseudo-papillary tumors
Insulinoma
Gastrinoma
VIPoma
Acinar cell carcinoma
Ductal adenocarcinoma
Mucinous cystic neoplasm
Lymphoma: Hodgkin lymphoma, large cell lymphoma, Burkitt's lymphoma
Germ cell tumors
Primitive neuroectoderma tumors: extraosseous Ewing's sarcoma
Mesenchymal tumors: rhabdomyosarcoma, leiomyosarcoma, schwannoma
Pediatric cancers evolving with pancreatic metastatic disease
Renal cell carcinoma
Sarcomas
Colorectal carcinoma
Neuroblastoma
Diseases (diagnosable) in pediatric patients predisposing to pancreatic cancer at adult age
Obesity
Diabetes mellitus, glucose metabolism disturbances and insulin resistance
Chronic and hereditary pancreatitis
Cystic fibrosis
McCune Albright syndrome
Multiple endocrine neoplasia type I
Von Hippel Lindau disease
Li Fraumeni syndrome

The pancreas may be also involved into neoplastic processes arising from the adjacent structures, such as lymphomas, teratomas, rhabdomyosarcomas and primitive neuroectodermal tumors. Secondary involvement may be seen during metastatic disease originating from neuroblastomas[4].

The study based on the data provided by the United States' National Cancer Institute for the Surveillance, Epidemiology and End Result (SEER) Database confirmed the scarcity of malignant pancreatic tumors in the pediatric population; data collected from a 30-year period of time identified only 58 patients aged under 20 years suffering from malignant pancreatic tumors[4].

Pancreatoblastoma

Pancreatoblastoma was described for the first time in a 15-mo-old male patient in 1957, and was originally termed "infantile adenocarcinoma of the pancreas"[5]. Later, histological similarities were found between the newly discovered type of tumor and the undifferentiated or incompletely differentiated fetal acini at 7-8 wk of gestation in the embryo, and the term "pancreatoblastoma" was imposed[6].

Most cases of pancreatoblastomas are sporadic but there have been cases of congenital nature associated with Beckwith-Wiedemann syndrome (macrosomia, macroglossia, visceromegaly, omphalocele and an increased risk for embryonal tumors, such as nephroblastoma, hepatoblastoma, rhabdomyosarcoma and mostly cystic pancreatoblastoma)[7].

Mostly encountered in pediatric patients aged 0 to 9-years-old, pancreatoblastomas are exceptionally rare in adults.

Pancreatoblastoma usually appears as a large well-defined solitary mass, with lobulated margins ranging between 1.5 cm and 20 cm, and with half of the cases arising from the cephalo-pancreatic region. It may present as a cystic mass accompanied by necrosis, especially when associated with Beckwith-Wiedemann syndrome[3].

Although it has been mostly cited in the male population, Rasalkar *et al*[8] reported two cases of pancreatoblastoma in 2 girls, aged 9-years-old and 11-years-old. The symptomatology included pain and discomfort in the epigastric region, accompanied by a local lump and food intolerance. In both cases, the mass had disordered the local anatomy by displacing the splenic vessels, right kidney, the renal veins and the inferior vena cava. The tumor was located on the head of the pancreas in the first case and on the distal body and pancreatic tail in the second case.

Other symptoms associated with pancreatoblastomas include weight loss, anorexia, fatigue, lethargy, and jaundice in rare cases. Local invasion has been cited, mostly involving the adjacent pancreas, biliary tree, and the local vessels, including the portal and the mesenteric veins. The most common sites of metastases are the liver and abdominal lymph nodes, but there have been case reports of rarely located metastasis, such as to the lungs, brain, omentum, colon, spleen and adrenal glands[9].

Solid pseudo-papillary tumors

Pseudo-papillary tumors of the pancreas are rarely encountered tumors of exocrine origin, having a strong female predilection and being diagnosed during childhood in half of the cases[3]. The literature suggests that they present no preference regarding location on the pancreas, a low-grade malignant potential and a favorable long-term prognosis after surgical treatment[10].

The histological origin of these tumors is not yet clear but it has been linked to ductal cells, endocrine or multipotent stem cells. One important issue is the possibility of recurrence and, hence, the mandatory imaging follow-up. Authors have reported cases of metastasis in the liver, regional lymph nodes, omentum, mesentery and peritoneum[11].

In a 15-year retrospective study, the authors found 11 patients with pseudo-papillary tumors presenting with abdominal pain or palpable abdominal mass, digestive intolerance, and 1 case of traumatic tumor rupture leading to hemoperitoneum; two cases were diagnosed incidentally during medical examinations for other diseases. In this lot, preoperative serum tumor markers were assessed as follows: α-fetoprotein, carcinoembryonic antigen, carbohydrate antigen (CA) 125 were within normal ranges, whereas elevated levels of CA 19-9 were found in 3 of 10 patients[10].

Insulinoma

Insulinomas are particularly rare in children, either the sporadic form or when associated with multiple endocrine neoplasia type 1 (MEN1). Although more than 90% of insulinomas are benign, there have been cases of malignant insulinomas, characterized by the World Health Organization (WHO) according to their metastatic potential and risk of recurrence and mortality. A tumor size larger than 2 cm as well as the presence of cytokeratin 19 and tumor staging and grading with Ki 67 > 2% are reported predictors for the metastatic disease. The most common sites of metastasis are the regional lymph nodes, the liver and bone[12].

The clinical manifestations include symptoms associated with neuroglycopenia, such as loss of consciousness, confusion, dizziness and lethargy, or with adrenergic stimulation, such as hunger, weight gain, tachycardia, palpitations and arterial hypertension; hypoglycemia may be difficult to recognize in neonates and small children up to 2 years of age because of the poor adrenergic symptoms. In these cases, the symptomatology associates with seizures, apnea and lethargy[13].

In a 20-year retrospective study, the fact that metastatic liver disease may appear years after the initial diagnosis and presumed curative surgical treatment of insulinomas was highlighted. In these cases, the metastatic liver disease manifested as recurrent, severe hypoglycemic episodes leading to coma[14].

Gastrinoma

Pancreatic gastrinomas are, in frequency, the second mostly encountered pancreatic neuroendocrine tumors (pNETs) after insulinomas. In children, this is an uncommon tumor, its diagnosis being based on complex biological and imagistic investigations. Because of its rarity, many cases of pediatric gastrinomas remain undiagnosed for

years; evidence of digestive ulcers associated with elevated levels of fasting gastrin is usually diagnostic for a gastrinoma associated with Zollinger-Ellison syndrome, but these findings have to be correlated with advanced imaging investigations in order to assess tumor localization and invasion. In the pediatric population, the malignancy rate is close to 30%, poor prognosis being associated with tumor size (> 3 cm), metastatic disease (especially hepatic, lymph nodes and bone metastasis), female gender, inadequate gastric hypersecretion control as well as the histopathological features[15].

According to the WHO classification, gastrinomas present with different potential of malignization corresponding to the degree of differentiation, as follows: Well differentiated gastrinomas with benign/uncertain behavior; well-differentiated gastrinomas with low-grade malignant behavior; and, poorly differentiated gastrinomas with high-grade malignant potential[16].

Massaro *et al*[17] reported a case of a gastrinoma with hepatic metastasis in a child investigated for Zollinger-Ellison syndrome who eventually underwent orthoptic liver transplant.

The symptomatology of sporadic pancreatic gastrinomas includes epigastric pain due to severe peptic ulcer disease, heartburn, chronic diarrhea, gastrointestinal (GI) bleeding, nausea and vomiting. There are pancreatic gastrinomas associated with *MEN1* mutation, which may manifest clinically at younger ages, before hyperparathyroidism; hence, complex management including assessment of ionized calcium and serum parathormone levels is important[16].

Vasoactive intestinal peptide secreting tumors

The vasoactive intestinal peptide secreting tumors (VIPomas) gather together a group of neuroendocrine tumors characterized by the secretion of the vasoactive intestinal peptide, resulting in the "WDHA" syndrome (featuring achlorhydria, hypokalemia and watery diarrhea). The literature suggests that 60%-80% of VIPomas have malignant potential and present with metastasis at first diagnosis. During childhood, VIPomas are usually diagnosed between 2-4 years of age, but there have been cases at younger age (Reindl *et al*[18] start their series with a 2-mo-old patient).

Metastasis are mostly located in the liver, but rare case of lymph node, lung and kidney metastasis have been described[19].

Acinar cell carcinoma

Carcinoma originating from the acinar cells represents a rare type of pancreatic cancer, although it is more frequently encountered during childhood compared to ductal cell adenocarcinoma. Even though the acinar cells represent the most commonly found cells in the pancreas, their malignant transformation accounts for only 1% of pancreatic exocrine tumors[20].

The histological differentiation between acinar cell carcinoma and pancreatoblastoma may be difficult in children, as their macroscopic and microscopic features tend to be similar. Other distinct characteristics include the presence of calcifications in one-third of the cases and the tendency for intratumoral hemorrhage[21].

Illyés *et al*[22] report the particular case of a 10-year-old male who presented with clinical and biological manifestations associated with Cushing's syndrome and was later diagnosed with pancreatic acinar cell carcinoma invading the retroperitoneum which evolved with multiple metastasis.

Ductal adenocarcinoma

Ductal adenocarcinomas are exceptionally rare entities in childhood. In spite of some case reports before 1993 and considering the current advancements in medicine and immunohistochemistry, these cases are no longer considered ductal adenocarcinomas [23].

The literature suggests that ductal adenocarcinomas appearing at a young age may present with a tendency for poor differentiation and a high degree of metastasis. The 5-year survival rate is similar to that in adults (2%-4%), although there have been authors who supported a lower survival rate due to delayed diagnosis in children and young adults[3].

Mucinous cystic neoplasms

Pancreatic cystic lesions usually present with a good prognosis in children, but rare cases of malignant (high grade dysplasia) or premalignant (low or intermediate grade dysplasia) course have been described. Recent studies suggest that mucinous cystic neoplasm is asymptomatic and discovered incidentally, and the malignant potential has to be considered, especially in tumors larger than 4 cm[24].

Lymphoma

Lymphomas may originate from the pancreatic tissue itself or may involve the pancreas with the true origin lying in the peripancreatic lymph nodes. During childhood, the most common type of lymphoma involving the pancreas is non-Hodgkin lymphoma; pancreatic and peripancreatic invasion can be seen in cases of large cell lymphoma and Burkitt's lymphoma. The pancreas involvement may be suggested by the presence of the mass itself or by a diffuse infiltration of the gland accompanied by local edema due to a process of acute pancreatitis or tumor lysis syndrome. There have been case reports of primary pancreatic lymphomas at a pediatric age manifested with obstructive jaundice, misleading the diagnosis towards a false acute cholestatic hepatitis[25,26].

Intrinsic pancreatic lymphomas usually affect female patients and manifest with nonspecific symptoms associated with the mass effect (abdominal pain, digestive intolerance). Imaging studies do not offer specific information and in order to make a definitive differential diagnosis from other pancreatic masses, a histological exam remains mandatory[27].

The literature reports less than 10 cases of pancreatic lymphoma manifesting as acute pancreatitis at debut. Athmani *et al*[28] report the case of a 6-year-old female with a rapidly progressive jaundice; in that case, abdominal imaging showed an important increase in the pancreatic size, homogeneous hepatomegaly and intra- and extrahepatic biliary ducts' dilatation. Ultimately, the histopathological examination set the diagnosis of Burkitt's lymphoma.

Standard diagnostic criteria for primary pancreatic lymphoma include absence of superficial lymphadenopathy, no enlargement of mediastinal lymph nodes visible on chest radiography, normal leukocyte count, the main mass located in the pancreas with peripancreatic lymph nodes involvement, and the absence of hepatic or splenic involvement[29].

Germ cell tumors

Extragonadal germ cell tumors represent 1%-3% of all childhood tumors. Even though their occurrence, originating from an upper abdominal organ, is exceedingly uncommon, they have been described on the head of the pancreas and causing biliary dilatation and mass-effect related symptoms. Based on the morphological characteristics and on the degree of differentiation, the WHO classifies teratomas as mature and immature[30].

Even though the malignant potential of pancreatic teratomas is yet to be established, 7%-10% of retroperitoneal teratomas with other localization are malignant[31]. For example, in other teratomas, the presence of yolk sac tumor microfoci may promote a malignant relapse after an incomplete surgical resection[32].

Primitive neuroectodermal tumors

Primitive neuroectodermal tumors usually originate in the bone and soft tissue, and there have been cases where they originated from solid organs containing neuroendocrine cells, like the pancreas, which accounts for 0.3% of primary pancreatic neoplasms at all ages[33].

In a literature review of extraosseous Ewing's sarcoma of the pancreas, Bose *et al*[34] pointed out the fact that the symptomatology usually associates with abdominal pain and jaundice; there were 2 particular cases in females of pediatric age where the tumor manifested with precocious puberty and 2 cases of GI bleeding and secondary iron deficiency anemia.

Primitive neuroectodermal tumors present an aggressive behavior, mostly due to the metastatic power; the most usual sites for metastasis are lungs, liver, bone and bone marrow.

Mesenchymal tumors

In children, the most common malignant mesenchymal tumor involving the pancreas is rhabdomyosarcoma. A percentage (specifically 15%) of rhabdomyosarcomas diagnosed during childhood originate from a site other than the head, neck, genitourinary tract and extremities. When situated in the abdomen, rhabdomyosarcomas can have originated from any part of the GI tract, including the extrahepatic biliary tract, pancreas and omentum; malignant ascites is an uncommon feature. An appreciable amount (specifically 14%) of these children present with metastatic disease at the initial diagnosis; the most common sites for metastasis are the lungs (36%), bone marrow (22%) and bones (7%)[35].

In rare cases, leiomyosarcomas may arise from the pancreatic duct and blood vessel walls within the pancreas; although rarely encountered, there have been cases of pancreatic leiomyosarcomas in children aged 14 and older[36].

Pancreatic schwannomas are rarely described in the literature, and more than 90% of them are benign tumors. However, there have been cases, mostly associated with von Recklinghausen disease, where the authors have described large schwannomas with malignant characteristics. Usually found on the pancreatic head and body, they usually manifest as nonspecific abdominal pain, weight loss, jaundice or GI bleeding [37].

Complete surgical resection is the treatment of choice in cases of pancreatic schwannomas, especially due to the fact that the character of malignancy cannot be established pre/during surgery and the fact that they do not respond to radiotherapy or chemotherapy[38].

Sheng *et al*[39] reported the first case of a low-grade malignancy pancreatic solitary fibrous tumor in a pediatric patient, a 14-mo-old male toddler.

PEDIATRIC CANCERS EVOLVING WITH PANCREATIC METASTATIC DISEASE

Metastatic pancreatic disease accounts for less than 2% of the entire pancreatic malignancies[40].

Pancreatic metastasis are rare entities that usually appear years after the diagnosis of the primary tumor. Following a topical literature review, authors reported that 62.6% of pancreatic metastasis appeared secondary to renal cell carcinoma, 7.2% secondary to sarcomas and 6.2% secondary to colorectal carcinoma[41]. Renal cell carcinoma is extremely rare in children, representing 1.8% to 6.3% of all types of renal cancers. Metastases originating from renal cell carcinomas usually present themselves as solitary or multiple masses inside the pancreas, which may occur even 20 years after the primary tumor manifestation. It is mandatory that the differential diagnosis of a pancreatic mass in patients with a history of renal cell carcinoma include secondary pancreatic metastasis[42].

Kim *et al*[43] reported the case of a 4-year-old male with stage 4 neuroblastoma, who presented with pancreatic metastasis manifesting as acute pancreatitis and rapidly progressive severe cholestasis. Farah *et al*[44] reported the case of a 15-year-old female who developed pancreatic metastasis due to an alveolar rhabdomyosarcoma located into the paranasal sinuses.

Pediatric pancreatic malignant tumors present with different degrees of aggressiveness and different potential for metastatic disease or local recurrences. The overall survival rates of pancreatic cancer diagnosed during childhood is not well established, but the 5-year survival rate for particular tumors ranges from more than 95% for some to as low as 2%-4% for ductal adenocarcinomas. For example, Bien *et al*[45] highlighted the fact that the 5-year survival rate of patients with pancreatoblastomas ranges between 30%-50%, despite surgical treatment and chemotherapy.

The 5-year survival rate for solid pseudo-papillary tumors has been reported between 95%-98% after complete surgical resection[46]. Primitive neuroectodermal tumors are highly aggressive and present with very poor prognosis; the 5-year survival rate is reported to be 50%[47].

Brecht *et al*[4] analyzed 5-year overall survival in a group of 228 patients under the age of 30 diagnosed with malignant pancreatic tumors (100 carcinomas, 85 endocrine tumors, 8 solid pseudopapillary neoplasms, 11 pancreatoblastomas). The data were extracted from the United States National Cancer Institute's SEER Public-use Database from 1973 to 2004. According to this study, 5-year overall survival (OAS) varied widely as follows: According to stage, it was 87%, 68%, 21 % for local (n = 54), regional (n = 42), distant metastatic disease (n = 108), respectively. According to histological types, OAS of patients with carcinoma was 33%, endocrine tumors 58%, solid pseudopapillary tumor of the pancreas 88%, pancreatoblastomas 66%. Following multivariate analysis, tumor stage, histology and age group are important and independent predictors for outcome.

Similar results were recently reported by Picado *et al*[48] analyzing the Data from the National Cancer Database (2004-2014) which included 109 children with pancreatic tumors. The 5-year overall survival by tumor histology was 95% for pseudopapillary tumors, 75% for neuroblastomas, 70% for pancreatoblastomas, 51% for endocrine tumors, 43% for sarcomas, and 34% for adenocarcinomas. On multivariable analysis, the strongest predictor of survival was the surgical resection[48].

Another recently published study, performed on 65 patients under the age of 21 diagnosed with pancreatic neoplasms, who followed the outcome after pancreatico-duodenectomy, reported that the 5-year overall survival by tumor histology was 95% for pseudopapillary neoplasm, 75% for neuroblastomas, 70% for pancreatoblastomas, 51% for endocrine tumors, 43% for sarcomas, and 34% for adenocarcinomas. In this study, the outcome was primarily associated with histology[49].

The burden of a diagnosis such as pancreatic cancer is often associated with adulthood, and pediatricians are tempted to overlook this possibility, especially in a toddler or young child. Diagnostic delays/mistakes are not uncommon at this age and they lead to worse outcome due to the aggressiveness of the local mass and the metastatic character.

DISEASES (DIAGNOSABLE) IN PEDIATRIC PATIENTS PREDISPOSING TO PANCREATIC CANCER AT ADULT AGE

Obesity

Various studies have highlighted a connection between increased body mass index (BMI) and the risk for pancreatic cancer[50-52].

It has been observed that a BMI of at least 30 kg/m^{2}was associated with a significantly increased risk of pancreatic cancer compared with a BMI of less than 23 kg/m^2[relative risk (RR): 1.72, 95%CI: 1.19-2.48], while an inverse relationship was also highlighted for moderate physical activity when comparing the highest to the lowest categories (RR: 0.45, 95%CI: 0.29-0.70), particularly among those with a BMI of at least 25 kg/m^2[53].

Moreover, it has been suggested that obese individuals develop pancreatic cancer earlier in life compared to patients with a normal weight, while also having lower survival rates following the diagnosis of pancreatic cancer[51,54].

Recent studies have also shown that overweight status and obesity in late adolescence and early adulthood are associated with an increased risk of this type of cancer[55,56].

Taking these aspects into consideration, researchers also questioned whether increased body weight during childhood would also be associated with adult pancreatic cancer. A prospective Danish cohort study including over 293000 individuals for whom data on height and BMI between the ages of 7 and 13 years were available, linked this information with data from Danish Cancer Registry. This study highlighted that increased BMI at every age from 7-13 years was significantly and positively associated with pancreatic cancer in adulthood, diagnosed up until age 70 [57].

Furthermore, the American National Institutes of Health-AARP (formerly known as the American Association of Retired Persons) Diet and Health Study Cohort and several pooled analyses of cohort studies also revealed that adolescent or early adulthood body size would be positively associated with pancreatic cancer[51,58].

BMI in children aged 7-13 was identified as being associated with the development of pancreatic cancer in adulthood[57].

These positive associations were not dependent of the evolution of BMI later in life, indicating that the risk was established to a certain extent in earlier life. Consequently, in the context of increased prevalence of childhood obesity, improved strategies for prevention and early management of childhood obesity should be considered to diminish the risk for developing pancreatic cancer in adulthood, but also for other various diseases including cardiovascular diseases and other types of neoplasia[59-61].

Diabetes mellitus, glucose metabolism, and insulin resistance

The relationship between diabetes and pancreatic cancer has been investigated extensively, since numerous epidemiologic studies have described an association between these two entities; a meta-analysis including 88 cohort and case-control studies identified an around 2-fold risk (RR: 2.08, 95%CI: 1.87-2.32) of pancreatic malignancy in diabetic patients compared to patients without diabetes[62-64].

However, the exact direction of this association is under debate, with some data suggesting that diabetes may be a consequence rather than a cause of pancreatic cancer, while others support the bidirectional relationship between the two entities, taking into account some pathophysiological background, such as the fact that insulin resistance, as well as obesity may be mediated by reduced levels of plasma adiponectin, a fat-derived hormone that has insulin-sensitizing and anti-inflammatory properties[65-67].

All these data have come from studies including adult populations and mainly focused on type 2 diabetes mellitus, with less data on type 1 diabetes mellitus. A systematic review of studies that assessed risk of pancreatic cancer in people with diabetes, specifically mentioning whether it was type 1 or type 2 diabetes, or a surrogate for type 1 diabetes, such as young age at onset, suggested that the risk of pancreatic cancer is comparably increased both for type 1 and type 2 diabetes. The results of this systematic review also underlined some causality within this relationship. With regard to type 2 diabetes, a bidirectional relationship can be called upon, meaning that insulin resistance and diabetes may be induced by undiagnosed cancer or precancerous conditions of the pancreas, but considering the increased risk of pancreatic cancer reported as 1.5- to 2-fold higher in type 2 diabetes even when impaired glucose tolerance is detected more than 10 years before onset of cancer, suggesting that reverse causality does not individually and exclusively explain the association[68,69].

With regard to type 1 diabetes, it is likely, considering the early age at its onset, that it precedes pancreatic cancer rather than the other way round; therefore, type 1 diabetes should be considered a risk factor for pancreatic cancer[70].

Large cohort studies indicated that cancer risk in type 1 diabetes is rather low, but other authors reported that this risk is comparable between type 1 and type 2 diabetes. As older studies report, type 1 diabetes associates an overall modestly increased cancer risk[71].

Both epidemiological and genetic studies have tried to prove the association between type 2 diabetes and pancreatic cancer. Pierce *et al*[72] analyzed the 37 risk alleles attributed to type 2 diabetes and found a significant positive association between 2 of these alleles and pancreatic cancer.

However, the currently available data is limited and further studies including large type 1 and 2 diabetes and nondiabetic cohorts are needed.

Chronic pancreatitis

Chronic pancreatitis is a risk factor for pancreatic adenocarcinoma, independent of etiology[73,74].

Although the exact pathophysiology is not yet clear, several signaling pathways have been identified as activated during pancreatic inflammation, in the context of repeated DNA damage, error-prone repair mechanisms, and the progressive accumulation of genetic mutations, ultimately stimulating the development of pancreatic cancer[75]. Pancreas precancerous histologic changes are associated with a sequential accumulation of genetic defects, namely pancreatic intraepithelial neoplasms, which are present in sporadic pancreatic adenocarcinomas and also in patients with a history of chronic pancreatitis. Mutations found in the early stages involve the *kRas* gene, followed by *p16/CDKN2A*, *TP53* and *SMAD4/DPC4* genes[76]. Mutations in all four genes have been recognized as driver mutations that trigger neoplastic transformation and tumor progression[77].

Non-hereditary chronic pancreatitis is also an important risk factor for pancreatic cancer[73,74]. Reports from the International Pancreatitis Study Group identified a cumulative risk of 1.8% at 10 years and 4% at 20 years for pancreatic cancer, independent of the etiology of chronic pancreatitis[78]. More recent data included in a meta-analysis has suggested that the risk of pancreatic cancer was elevated 16-fold in patients with more than 2 years since their diagnosis of chronic pancreatitis, and even though this risk declines over time, it persists even after long-term follow-up. After at least 9 years of follow-up from the time of diagnosis of chronic pancreatitis, this patient category still had an over 3-fold increased risk of pancreatic cancer compared to patients without chronic pancreatitis[79].

Although these data were obtained from an adult population, they raise concern regarding the pediatric population, especially when considering patients with hereditary pancreatitis. Pancreatitis is rare in the pediatric population and frequently reported as idiopathic pancreatitis, showing gaps in the identification of the etiology. Recently, it was shown that genetic factors play a far more important role in the development of chronic pancreatitis in infancy and childhood than previously thought [80].

Hereditary pancreatitis is defined as two first-degree relatives or at least three second-degree relatives in two or more generations with chronic pancreatitis for which there is no other etiology. Several mutations have been associated with hereditary pancreatitis, from the initial description of the mutation in the cationic trypsinogen gene (*PRSS1*) by Whitcomb *et al*[81] to mutations in the *SPINK1*, *CPA1* and *CFTR* genes[82-85].

Due to a lack of specific signs and even to a sometimes quiet presentation, hereditary pancreatitis is frequently diagnosed at an advanced stage[86].

In some sporadic cases, mutations compatible with hereditary pancreatitis can be found without a corresponding family history, which could be explained by inheritance from unaffected carrier parents or even spontaneous *de novo* mutations[75,87].

Although progress has been made in diagnosing hereditary pancreatitis, the therapeutic pathway for this entity in children is still under debate[80]. Furthermore, counseling in patients with hereditary pancreatitis and their families should cover several aspects, from highly penetrant childhood-onset disease to a predisposition for pancreatic cancer in adulthood[88].

Studies are suggesting that *PRSS1* and *CFTR* gene mutations may not be directly linked with the development of pancreatic cancer. The onset of hereditary pancreatitis during childhood or early adulthood favors the perpetual exposure to chronic inflammation. The link between inflammation and cancer may not be limited to a subset of tumors but may be present within different types of cancer including lung, bladder, digestive, skin, and vulvar cancer. Hence, even though mutations in the *PRSS1* and *CFTR* genes are not directly associated with pancreatic cancer, chronic inflammation may favor tumor formation[89].

Beyond hereditary pancreatitis, other genetically determined conditions, including exocrine pancreatic insufficiency could evolve towards chronic pancreatitis and therefore potentially associate an increased risk for pancreatic cancer. Among these, Shwachman-Diamond syndrome is included. This a rare multi-organ recessive disease, mainly characterized by bone marrow failure leading to increased risk of transformation to myelodysplastic syndrome, skeletal defects, short stature, and pancreatic insufficiency contributing to the failure to thrive[90].

Cystic fibrosis

The cystic fibrosis phenotype results from mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator protein encoded by the *CFTR* gene and is among the most common autosomal recessive disease in White people having an increased risk of pancreatic cancer[91].

A meta-analysis investigating the risk for various cancers in cystic fibrosis patients identified a standardized incidence ratio of 6.18 (95%CI: 1.31-29.27), which is 2-fold to 5-fold higher in patients who had undergone lung transplantation in this setting[92]. Since there is an excess risk of early-onset pancreatic cancer in patients with cystic fibrosis, interventions for avoiding this setting should be identified. These could range from tackling environmental factors to gene therapy targeting mucin genes. Considering that nutritional deficiencies represent another possible risk factor for cancer, appropriate nutritional interventions to avoid or correct deficits should also be considered[93].

Mccune-Albright syndrome

McCune-Albright syndrome (MAS) represents a rare disorder, characterized by variable phenotypic expressions, including fibrous dysplasia of bone, hyperfunctioning endocrinopathies, and café-au-lait macules, in the context of a somatic gain-of-function mutation in the *GNAS gene*, which encodes the cAMP-regulating protein $G\alpha_s$, taking place early in embryonic development[94]. These types of *GNAS* mutations were previously identified in various types of neoplasias (pituitary, thyroid, appendiceal, and gonadal tumors, and breast cancer)[95-99].

Since there is such a wide range of pathology associated with MAS, it is essential to define them all and to establish an optimal screening strategy and clinical management. The activating *GNAS* mutations have been identified as somatic driver mutations in sporadic intraductal papillary mucinous neoplasms (IPMNs) and various GI lesions, also suggesting a potential role in pancreatic and GI tumorigenesis[100].

In a cohort of patients with MAS, about 46% developed IPMNs, which have risk for malignant degeneration[100,101]. This can lead to IPMN-associated adenocarcinoma but also to developing concurrent or distinct ductal adenocarcinoma, a pattern which has been described in a 2%-9% of patients who are being followed for IPMN[102,103].

Consequently, GI manifestations in MAS are common and include precursory pancreatic lesions for pancreatic cancer; therefore, patients with MAS may benefit from evaluation for GI pathology. Even though the optimal care for pancreatic lesions in MAS is not clearly shaped, a thorough GI history-taking at every visit and consideration of imaging with abdominal magnetic resonance imaging/magnetic resonance cholangiopancreatography would be useful[100].

A small observational study describes the link between MAS and pancreatic cancer, liver adenoma and choledochal cysts *via* the cAMP pathway. Taking into consideration this association, the authors suggest that all patients suffering from MAS should benefit from routine screening by magnetic resonance imaging (MRI) and if no lesion is observed, surveillance MRI may be performed every 5 years[104].

MEN1

MEN1 mutations cause a rare hereditary tumor syndrome, with an autosomal dominant transmission and high degree of penetrance. MEN1 tumors are caused by inactivating mutations of the tumor suppressor gene *MEN1*, and is characterized by a predisposition to a multitude of endocrine and nonendocrine tumors[105]. The classical phenotype includes hyperplasia and/or tumors of parathyroid, enteropancreatic, and/or anterior pituitary origin, with 30%–70% of patients developing enteropancreatic tumors, either functional or non-functional pancreatic endocrine pancreatic tumors upon reaching middle age[106]. Germline *MEN1* mutations have also been noted in families with only a parathyroid disorder, namely familial isolated primary hyperparathyroidism, but this type of mutation has also been reported in some cases of "sporadic" pNETs[107-109].

Considering the pattern of transmission and variety of manifestations, the Endocrine Society's clinical practice guidelines for MEN1 recommend a complex surveillance scheme commencing at early pediatric age, aiming to detect early and manage optimally MEN1-associated manifestations and tumors[110]. Recommended screening for pancreatic tumors in this setting begins early, at age 5 for insulinoma, with recommendation for yearly evaluation of fasting glucose and insulin level; for other pNETs, screening is recommended beginning at age 10, with evaluation of chromogranin A, pancreatic polypeptide, glucagon, vasoactive intestinal peptide levels and annual imaging, comprising magnetic resonance imaging, computed tomography or endoscopic ultrasound, considering that large pancreatic tumors may develop between 10 and 20 years of age[105].

Von Hippel-Lindau disease

Von Hippel–Lindau (vHL) disease is an autosomal dominant syndrome which occurs secondary to germline mutations in the *VHL* tumor suppressor gene, with patients being at risk of developing visceral cysts and tumors throughout the body[111]. Most commonly, the pancreas and kidneys are involved, with development of cystic lesions, but there is also an increased risk of developing neoplasms, often with clear cell features, including cerebellar and retinal hemangioblastomas, adrenal pheochromocytomas, clear cell renal cell carcinomas, pancreatic neuroendocrine tumors and pancreatic serous cystadenomas.

Although the most frequent pancreatic lesions include pancreatic cysts, which are found in 75% of vHL patients, one-fifth of the vHL patients will present pNETs, arising earlier than in non-syndromic patients, mostly in the fourth decade of life[112, 113].

Considering the increased potential for metastatic disease of pNETs in vHL patients and also the risk for metastasis generally associated with pNETs, regardless of the histologic grade, surgical resection is typically recommended for all pNETs greater than 2 cm[114]. However, taking into account the possibility of recurrent pNETs in vHL patients, in the context of the particular genetic background, the therapeutic decision must be well considered[115]. Although these lesions most commonly present during adulthood, screening and surveillance should begin in the pediatric years for patients with vHL disease.

Li Fraumeni syndrome

Li Fraumeni syndrome (LFS) is inherited in an autosomal dominant manner, which implies an increased risk for multiple primary cancers in affected individuals[116]. The most frequently implied cancers include bone and soft tissue sarcomas, leukemia, breast cancer, and brain tumors; subsequent evidence highlighted a broader spectrum of neoplasia, including cancers of the lung, digestive tract, prostate, ovary and pancreas, as well as lymphoma and melanoma[117,118].

Germline mutations in the tumor suppressor gene *TP53* are mainly incriminated for this syndrome; however, the absence of detectable germline *TP53* mutations in some LFS families suggests other genes are potentially involved in this syndrome[119].

There are various diagnostic criteria for the syndrome, such as the one included in the classical definition, which was afterwards extended for Li Fraumeni-like syndrome (an entity which shares some features of LFS but does not meet the strict LFS diagnostic criteria), in order to establish the criteria for *TP53* genetic testing[120]. Since

there is such a diversity of types of neoplasia which could arise in the context of LFS, we currently lack appropriate clinical surveillance strategies in this setting. Moreover, in the absence of a thorough knowledge on potential clinical benefits, psychosocial and economic impact of a comprehensive clinical surveillance protocol on early cancer detection, including pancreatic cancer screening, in asymptomatic *TP53* mutation carriers remains unknown.

OTHER GENE MUTATIONS ASSOCIATED WITH A PREDISPOSITION FOR PANCREATIC CANCER

Genetic risk factors play a major role in the development of pancreatic cancer, even if we are considering hereditary pancreatic cancer, familial pancreatic cancer or cases of pancreatic cancer associated with a familial cancer syndrome. The genetic predisposition for developing a particular malignancy has been of interest over the last decades, and several gene variants have been linked to an increased risk for pancreatic cancer, such as *BRCA1* and *BRCA2, STK11, PRSS1, PALB2, ATM, CDKN2A, APC, MLH1, MSH6, MSH2,* and *PMS2*[121].

However, this area of genetic research is still providing new information with the expectation to develop tests in order to assess the individual risk factors for pancreatic cancer, especially inside families with pancreatic cancer patients.

The definition of familial pancreatic cancer implies the existence of at least one pair of first-degree relatives suffering from pancreatic cancer, in the absence of a particular syndrome inside the family[122].

BRCA1 and *BRCA2* mutations are highly associated with inherited breast and ovarian cancer, but in carriers other types of cancer occur at higher rates than in the general population, including of pancreatic cancer. Brose *et al*[123] emphasize the fact that the general population risk of 1.3% transforms into a 2.8 RR for pancreatic cancer in people who carry the *BRCA1* mutation.

Familial atypical multiple mole melanoma syndrome is disease related to a cell cycle regulator gene for the p16 protein product, CDKN2A. Screening should begin early in childhood, in order to capture the early debut of both malignant melanoma and pancreatic cancer[124].

APC gene mutations are associated with the onset of familial adenomatous polyposis, a condition which, classically, increases the risk for colorectal cancer. Nowadays, evidence strongly suggests a correlation between inherited mutation of this gene and duodenal, thyroid, hepatic and pancreatic cancers[125].

The hereditary non-polyposis colorectal cancer known as Lynch syndrome also accounts for an increased risk for pancreatic cancer (1.3%-4%)[126]. Geary *et al*[127] pointed out the 30-fold increased risk for pancreatic cancer in patients with particular germline mutations in genes associated with the appearance of Lynch syndrome, such as *MLH1, MSH2, MSH6* and *PMS2.*

Complete family medical history is of paramount importance in assessing the particular risk for pancreatic cancer. In risk stratification, early molecular studies and germline mutation testing are mandatory.

CONCLUSION

Pancreatic cancer is generally considered an adulthood condition. In spite of this fact, its origins may lay in diseases or predisposing conditions which may be diagnosed at an early age, hence the need for rigorous screening strategies in patients with hereditary diseases connected with pancreatic cancer or familial aggregation of such conditions. Clinicians should be aware of rare conditions diagnosed during childhood, which may associate an increased risk for adult-onset pancreatic cancer. Also, the rare diagnosis of pancreatic cancer should be taken into consideration even at a pediatric age, especially when facing previously considered idiopathic acute pancreatitis or cholestatic hepatitis. The clinical features associated with pancreatic or peripancreatic masses may mimic other diseases and their management should include extensive imaging and histopathological examinations.

Malignant pancreatic tumors remain extremely rare in children and young adults and limited data on incidence and outcomes are available. Entities change over the age groups towards a predominantly increased number of carcinomas with worse outcome in older patients. The overall survival of pediatric patients with pancreatic

tumors is grim, and the survival rates tend to vary between different tumors. Given the rarity and aggressivity of many histological types of pancreatic tumors in children, in many instances it is difficult to assess whether there is any link between pancreatic cancer in childhood and adulthood.

International collaboration is needed to learn more about pediatric pancreatic tumors and to study the link between pancreatic tumors in childhood and adulthood.

Prophylaxis strategies should include weight control during childhood, screening for diabetes mellitus at younger ages, early routine imagistic investigations in cases with a predisposition towards pancreatic cancer or pancreatic metastasis. MRI and endoscopic ultrasonography are the recommended screening methods for pancreatic lesions. Also, screening strategies should include parents, siblings and children of patients with pancreatic cancer, especially in cases where there are more than 2 pancreatic cancer patients within the same biological family.

The implementation of transition programs from pediatric to adult healthcare services remains mandatory for these patients, namely childhood pancreatic cancer survivors or children with predisposing conditions for pancreatic malignancies.

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MINIREVIEWS

Application of artificial intelligence in preoperative imaging of hepatocellular carcinoma: Current status and future perspectives

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Abstract

Hepatocellular carcinoma (HCC) is the most common primary malignant liver tumor in China. Preoperative diagnosis of HCC is challenging because of atypical imaging manifestations and the diversity of focal liver lesions. Artificial intelligence (AI), such as machine learning (ML) and deep learning, has recently gained attention for its capability to reveal quantitative information on images. Currently, AI is used throughout the entire radiomics process and plays a critical role in multiple fields of medicine. This review summarizes the applications of AI in various aspects of preoperative imaging of HCC, including segmentation, differential diagnosis, prediction of histo-pathology, early detection of recurrence after curative treatment, and evaluation of treatment response. We also review the limitations of previous studies and discuss future directions for diagnostic imaging of HCC.

Key Words: Hepatocellular carcinoma; Radiomics; Artificial intelligence; Diagnosis; Treatment; Recurrence

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Core Tip: Hepatocellular carcinoma (HCC) threatens human health because of its high morbidity and recurrence rates. Patients with HCC may benefit from early diagnosis,

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timely treatment, and appropriate follow-up strategies. In the era of big data, artificial intelligence (AI) provides critical information regarding the diagnosis, treatment, and prognosis of HCC. We herein discuss the role of AI in the following aspects of preoperative imaging: Segmentation, differential diagnosis, prediction of histopathology, early detection of recurrence after curative treatment, and evaluation of treatment response.

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INTRODUCTION

Hepatocellular carcinoma (HCC), which most often arises from chronic hepatitis B virus infection, is one of the main causes of cancer-related deaths worldwide, particularly in China[1]. HCC presents with characteristic radiological features and can be diagnosed without biopsy. Imaging is therefore crucial for diagnosis and management. Computed tomography (CT) is the most widely used method for HCC diagnosis, although magnetic resonance imaging (MRI) is the optimal diagnostic modality, owing to its multi-parameter imaging techniques. However, even with the application of dynamic contrast-enhanced MRI (DCE-MRI), the imaging diagnosis of HCC is challenging because of atypical imaging manifestations and liver tumor diversity.

A single clinical image contains a large amount of quantitative information that can provide crucial data for diagnostic and treatment purposes. This information can be processed using innovative methods. Radiomics has recently gained attention for its potential to further analyze images. It allows for the extraction of a large amount of quantitative objective data included in radiological images that could be explored for determining underlying biological processes[2]. The workflow of a radiomics study generally includes five stages: Image acquisition, segmentation, feature extraction, exploratory analysis, and modeling[3] (Figure 1). Every stage is closely related to artificial intelligence (AI). The concept of AI was first advocated in 1955 by McCarthy *et al*[4], who described AI as a computer program that attempts to simulate human cognitive functions. AI can learn and solve problems to improve itself.

Machine learning (ML) is at the core of AI and involves various techniques, such as artificial neural networks, support vector machines (SVM), and random forest (RF). Deep learning (DL) is an important branch of ML, and convolutional neural networks (CNNs) are a type of DL algorithm. The relationships among AI, ML, and DL are described in Figure 2. With technological advances and the use of AI, radiomics has rapidly developed in recent years, and all radiomics techniques have now been utilized in studies within the medical field. This review focuses on the application of AI for HCC imaging, including segmentation, differential diagnosis, prediction of histopathology, early detection of recurrence after curative treatment, and evaluation of treatment response.

SEGMENTATION OF HCC LESIONS

Although HCC segmentation is challenging, it is crucial for various medical imaging analyses. Manual and semi-manual segmentation are time-consuming and susceptible to interobserver variability, which might in turn lead to the biased results. In contrast, automatic segmentation based on an AI algorithm is more repeatable and efficient. CNNs have been successfully applied for the automatic segmentation of hepatic lesions in both CT and MRI. Chlebus *et al*[5] figured out a CNN model for the automatic segmentation of liver tumors on CT; however, with respect to tumor detection, the model performed poorly compared with human performance. Bousabarah *et al*[6] established a DL algorithm to automatically delineate the liver and HCC lesions on MRI. The model was a combination of a deep CNN, RF, and thresholding. The mean dice similarity coefficient between the DL model and manual



Figure 1 The five stages of a radiomics study where artificial intelligence can play a role.

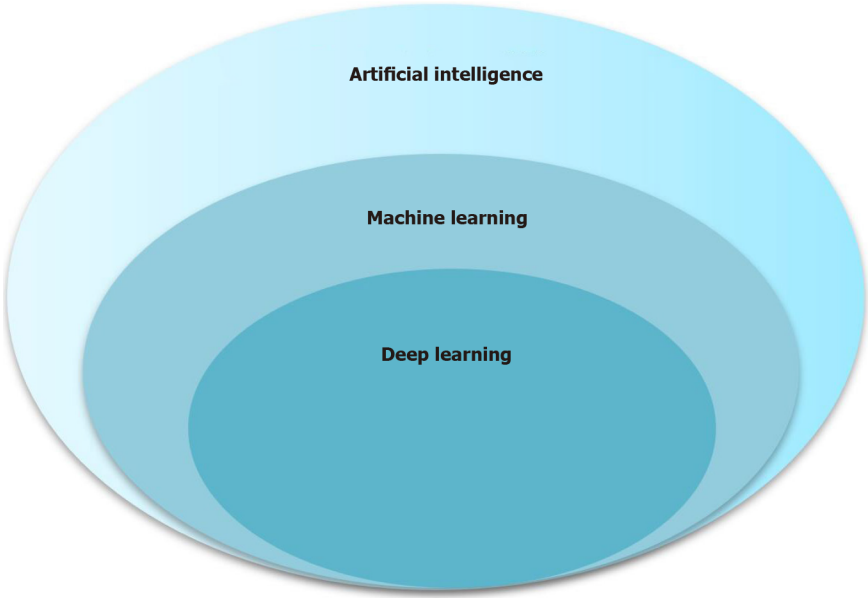


Figure 2 Relationship of artificial intelligence, machine learning, and deep learning.

segmentation was 0.64/0.68 (validation/test) for tumor segmentation and 0.91/0.91 for liver segmentations. Current methods do not achieve a comparably high level of performance in liver imaging for automatic tumor segmentation. This may be due to the heterogeneity of the liver parenchyma and the tumor itself. Larger datasets will be required to improve the accuracy of DL algorithms and will ensure optimal sensitivity and specificity.

DIFFERENTIAL DIAGNOSIS

Imaging-based differential diagnosis improves the accuracy of diagnosis. For HCC, the primary diagnostic modalities are CT and MRI. Radiomics with AI has been gradually applied in clinical research, as it can provide quantitative information, including additional differential characteristics not yet recognized in current radiological diagnosis. This could, in turn, help less-experienced radiologists diagnose focal liver lesions more accurately. Table 1 summarizes the studies on the differential diagnosis of HCC with AI.

CT

Some studies have applied conventional ML algorithms for the differentiation of liver masses. In the non-cirrhotic liver, Nie *et al*[7,8] reported CT-based radiomics nomograms for the preoperative differentiation of HCC from hepatocellular adenoma (HCA) and focal nodular hyperplasia (FNH). It showed good discrimination capability, with an area under the curve (AUC) of 0.96/0.94 and 0.979/0.917 (training/validation) for differentiating HCC from HCA and FNH, respectively In patients with cirrhosis, ML techniques could classify indeterminate liver nodules as HCC or non-HCC based on triphasic CT scans, with an AUC of 0.70/0.66 (training/validation)[9]. Ponnoprat *et al*[10] developed an ML method to differentiate between HCC and intrahepatic cholangiocarcinoma (ICC), the two most common malignant liver tumors, based on multi-phase CT. They reported an 88% accuracy in HCC-ICC classification. DL with CNN for the differential diagnosis of liver lesions also showed relatively high diagnostic capability, reaching an AUC of 0.86-0.99[11-13].

Table 1 Summary of studies on differential diagnosis of hepatocellular carcinoma

Ref.	Aim of the study	Modality	Patients	Method	AUC of the final model
Nie *et al*[7]	Differentiation between HCC and HCA	CT	131	ML	T: 0.96, V: 0.94
Nie *et al*[8]	Differentiation between HCC and FNH	CT	156	ML	T: 0.979, V: 0.917
Mokrane *et al*[9]	Differentiation between HCC and non-HCC nodules	CT	178	ML	T: 0.70, V: 0.66
Ponnoprat *et al*[10]	Differentiation between HCC and ICC	CT	257	ML	NA
Shi *et al*[11]	Differentiation of HCC from other FLLs	CT	342	DL	0.925
Yasaka *et al*[12]	Liver mass classification	CT	560	DL	0.92
Cao *et al*[13]	FLL classification	CT	375	DL	V: 0.88-0.99
Jiang *et al*[14]	Comparing the diagnostic accuracies of EASL (v2018), LI-RADS criteria, and radiomics models for HCC	MRI	211	ML	T: 0.861, V: 0.810
Zhen *et al*[15]	Classification of liver tumors	MRI	1411	DL	0.963-0.998
Liu *et al*[16]	Differentiation of cHCC-CC from CC and HCC	MRI	85	ML	0.77
Huang *et al*[17]	Diagnosis of DPHCC	MRI	100	ML	0.784
Jian *et al*[18]	Characterization of HCC	MRI	112	DL	NA
Wu *et al*[19]	Classification of HCC and hepatic hemangioma	MRI	369	ML	T: 0.86, V: 0.89

T: Training cohort; V: Validation cohort; NA: Not available; AUC: The area under the receiver operating characteristic curve; ML: Machine learning; DL: Deep learning; HCC: Hepatocellular carcinoma; HCA: Hepatocellular adenoma; FNH: Focal nodular hyperplasia; ICC: Intrahepatic cholangiocarcinoma; cHCC-CC: Combined hepatocellular cholangiocarcinoma; CC: Cholangiocarcinoma; DPHCC: Dual-phenotype hepatocellular carcinoma; FLL: Focal liver lesion.

MRI

MRI can provide more comprehensive information for differential diagnosis than CT because of its multi-parameter techniques and various tissue contrast mechanisms. Accurate diagnosis of HCC remains a challenge because of liver tumor diversity and relies on the experience of radiologists. Jiang *et al*[14] established a radiomics signature from multi-sequence MRI using the least absolute shrinkage and selection operator (LASSO) model and multivariate logistic regression analysis. The AUC of the radiomics signature was 0.810. It showed a comparable accuracy with the LI-RADS (0.841) and EASL criteria (0.811) for HCC diagnosis. Zhen *et al*[15] used CNNs to develop a DL model to classify liver tumors based on MR images. The CNN model combined with clinical data performed well in identifying HCC (AUC, 0.985; 95%CI: 0.960-1.000), metastatic tumors (AUC, 0.998; 95%CI: 0.989-1.000), and other primary malignancies (AUC, 0.963; 95%CI: 0.896-1.000). Some researchers have also applied ML algorithms to the differential diagnosis of primary liver cancer subtypes. Liu *et al*[16] reported an ML analysis of MRI radiomics features for the differentiation of combined hepatocellular cholangiocarcinoma (cHCC-CC) from HCC and cholangiocarcinoma. The model showed acceptable performance, with an AUC of 0.77. Huang *et al*[17] created a radiomics signature from Gd-EOB-DTPA-enhanced MRI for the diagnosis of dual-phenotype HCC (DPHCC). They used LASSO and four classifiers: Multi-layer perceptron, SVM, logistic regression, and K-nearest neighbor. The combination of different phases and classifiers achieved the best performance in the preoperative diagnosis of DPHCC. ML and DL algorithms can also be used in non-contrast MRI to increase the diagnostic accuracy of HCC[18,19]. This is beneficial for patients who cannot receive contrast injections.

CT- or MRI-based ML and DL models have demonstrated promising predictive performance and have reached a high level of accuracy similar to that of experienced radiologists. Accurate preoperative diagnosis of focal liver lesions can help clinicians make proper decisions, optimize patient management, and improve patient prognosis.

PREOPERATIVE PREDICTION OF HISTOPATHOLOGY

Grade

Histological grade represents the biological behavior and aggressiveness of tumors. The high recurrence rate of HCC is associated with its pathological grade. The pathological grade of HCC determined using imaging may provide valuable prognostic information. Mao *et al*[20] developed an ML model based on contrast-enhanced CT for preoperative prediction of the pathological grade of HCC. The model achieved an AUC of 0.8014 when combined with clinical factors. The radiomics signature based on T1WI, T2WI, and DCE-MRI was also found to be significantly related to the histopathological grade of HCC[21,22]. Yang *et al*[22] proposed a DL model with a multichannel fusion three-dimensional CNN (3D-CNN) based on DCE-MR to differentiate among pathological grades. The model reached an average accuracy of 0.7396 ± 0.0104. Thus, further improvements are needed to achieve better diagnostic performance of radiomics models for histological grade.

Microvascular invasion

In addition to predicting the HCC grade, the prediction of microvascular invasion (MVI) has also become a common topic of study. MVI status is a crucial factor influencing treatment selection and follow-up planning; thus, it must be determined preoperatively[23,24]. Some studies have tried to predict MVI before surgery using radiological images and clinical factors. They found that MVI was closely related to several factors. Some studies used LASSO to construct radiomics signatures from CT and MRI and developed models with various conventional ML algorithms for the preoperative prediction of MVI[25-27]. The results showed favorable predictive accuracy for MVI status in HCC patients, especially when combined with clinical factors[28,29]. Jiang *et al*[30] figured out a CT-based model using eXtreme Gradient Boosting and DL algorithm with 3D-CNN to predict MVI preoperatively. The 3D-CNN model demonstrated high diagnostic capability, with an AUC of 0.980/0.906 (training/validation). However, different dimensionality reduction and modeling methods affect the diagnostic performance of the final models. Ni *et al*[31] retrospectively analyzed 206 HCC cases to explore the best radiomic-based diagnostic model. The LASSO + GBDT method showed better performance than the other methods, when the threshold probability was more than 0.22.

MVI usually occurs in the peritumoral region, but it is unclear whether including the features of the peritumoral region could improve predictive capability. Nebbia *et al*[32] developed an ML model for preoperative prediction of MVI status using multiparametric MRI. They found that radiomics features extracted from the tumor region had good diagnostic performance, with an AUC of 0.867. Radiomics features from the peritumoral region also showed an association with MVI; however, the AUC was slightly lower than that of intratumoral radiomics features. Although peritumoral enhancement pattern is reported to be a good predictor of MVI status[33], the usefulness of the features extracted from peritumoral regions for predicting MVI status needs further evaluation in larger populations.

Molecular profiling

HCC originates from hepatocytes and/or hepatic progenitor cells and can express various molecular phenotypes[34]. As such, prognosis varies even among patients with the same pathological grade. Molecular profiling is an important modality that may reflect the biological behavior and invasiveness of tumors. Several studies have used ML methods to identify the molecular phenotypes of HCC preoperatively. CK19 is a biliary-specific marker, and CK19 positivity is associated with a poor postoperative prognosis in HCC[35,36]. In a recent study, the radiomics signature derived from gadoxetic acid–enhanced MRI was used to predict CK19 status in HCC. The final model combined the radiomics signature and clinical factors and achieved a sensitivity of 0.818/0.769 (training/validation) and specificity of 0.974/0.818 (training/validation)[37].

Glypican 3 (GPC3), a type of heparan sulfate proteoglycan, is located on the cell surface. Previous studies have found that GPC3 is closely associated with postoperative metastasis and recurrence in patients with HCC[38]. Furthermore, GPC3 is considered a potential immunotherapeutic target for HCC therapy, especially in patients with unresectable HCC. Gu *et al*[39] constructed a radiomics signature from MRI and achieved good predictive efficacy, with an AUC of 0.879/0.871 (training/validation). Combining the radiomics signature with α-fetoprotein levels further improved the predictive performance, with an AUC of 0.926/0.914 (train-

ing/validation). Another molecular biomarker of HCC is the Ki-67 index. A high Ki-67 index is related to early recurrence and poor prognosis, thus making it a potential indicator of tumor aggressiveness. Recent studies have reported that the Ki-67 status can be predicted preoperatively using CT and MRI[40,41]. Prediction of molecular phenotypes *via* preoperative imaging can aid in the selection of individualized therapeutic strategies.

PREDICTION OF TREATMENT RESPONSE AND EARLY RECURRENCE AFTER CURATIVE TREATMENT

Individualized medical care depends on accurate risk stratification systems. These systems help select the proper treatment and evaluate treatment response. Pretreatment imaging acts as an important role in predicting the effects of treatment, helping clinicians choose the best individualized treatment strategy for patients.

Early recurrence, that is, recurrence within 1-2 years after resection or ablation, is a strong influencing factor of poor prognosis in patients with HCC. A radiomics nomogram derived from preoperative CT and MRI was established to predict early recurrence after surgery or curative ablation in HCC patients[42,43]. The radiomics nomogram comprising both the radiomics score and clinicoradiological risk factors demonstrated good performance, with an AUC of 0.785-0.844[44,45]. Prediction of early recurrence is critical for planning follow-up surveillance strategies and determining the necessity of further interventions after curative treatment. Wang *et al*[46] proposed a DL-based radiomics approach from multi-phase CT images to predict the early recurrence of HCC; it achieved an AUC of 0.825. Unfortunately, image-based radiomics models for early recurrent HCC have poor reproducibility among medical centers[47], limiting their application in clinical practice.

GUIDANCE OF TREATMENT SELECTION

Trans-arterial chemoembolization

Patients with unresectable HCC can undergo trans-arterial chemoembolization (TACE), local radiofrequency ablation, and systemic treatment with sorafenib. ML or DL models based on pretreatment CT or MRI have been recently considered as potential tools for predicting treatment response to TACE for HCC[48,49]. Morshid *et al*[50] used pretreatment quantitative CT image features and clinical factors to develop an ML model to predict treatment response to TACE; the model had an accuracy of 74.2%. Abajian *et al*[51] used MR imaging and clinical data to create an AI model for the prediction of TACE treatment response; the model had an overall accuracy of 78%. Similar results were obtained in other studies, confirming the usefulness of radiomics features extracted from pretreatment CT or MRI for the prediction of tumor response to TACE[52-54]. Peng *et al*[55] described a DL model of a residual CNN to predict the treatment response to TACE for HCC. The final model had a high accuracy in predicting four different therapy response types (complete response, partial response, stable disease, and progressive disease). Thus, it could help clinicians identify patients who will optimally benefit from TACE.

Immunotherapy

Immunotherapy has been shown to be a promising treatment for HCC; however, treatment response to immunotherapy remains low[56-58]. Therefore, it is necessary for clinicians to identify which patients will respond to immunotherapy. Treatment response to immunotherapy is highly dependent on the immune status of the tumor [59]. Tumor immunoprofiling is thus important in predicting its effect. A contrast-enhanced CT-based Rad score developed using the ML algorithm showed high predictive power for $CD8^+$ T-cell infiltration, which is associated with the immunotherapy response[60]. Hectors *et al*[61] reported that MRI radiomics features were correlated with various immunohistochemical cell markers and the expression of certain immunotherapy targets. Chen *et al*[62] used clinical data and intratumoral and peritumoral radiomics features to build an ML model from Gd-EOB-DTPA-enhanced MRI. The model showed excellent performance in predicting the immunoscore, with an AUC of 0.926 (95%CI: 0.884-0.967). Collectively, these findings indicated that radiomics features extracted by ML methods might serve as noninvasive predictors of the immune characteristics of HCC and assist physicians in identifying patients who

will benefit from immunotherapy.

LIMITATIONS

The application of AI in different fields of medical research has demonstrated promising results; however, there are some limitations. First, nearly all the research was retrospective and included a relatively small sample size. Therefore, the performance of these predictive models should be validated in larger, multicenter, and prospective studies. Second, the predictive models had limited reproducibility for application in clinical practice, and image heterogeneity might be a significant influencing factor. Third, the AI calculation algorithm requires specialized software packages, leading to increased medical costs. The workflow includes imaging acquisition, segmentation, feature extraction, exploratory analysis, and modeling, making it complex and diverse, further limiting its clinical application.

While AI and radiomics have proven useful in various aspects of HCC, the underlying mechanisms have not been clearly stated, such as pathological correlation and relationship between radiomics and genomics. More research is needed to explore the relationships among imaging, pathophysiology, and prognosis.

CONCLUSION

AI has been applied in many studies on preoperative imaging of HCC. It can extract a large amount of quantitative information from images and reflect pathophysiological processes. Diagnostic and predictive models using AI algorithms have demonstrated promising results in the fields of segmentation, differential diagnosis, prediction of histology, and guidance for treatment selection. However, considering the limitations and complexity of AI, additional research is needed before it can be widely used in clinical practice. Some specific issues, such as reproducibility, heterogeneity of imaging acquisition, and lack of external multicenter validation, need to be considered. Further research will be crucial in improving the accuracy and reproducibility of diagnostic and predictive models, enabling their application for individualized treatment in patients with HCC.

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MINIREVIEWS

Artificial intelligence application in diagnostic gastrointestinal endoscopy - Deus ex machina?

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Abstract

The close relationship of medicine with technology and the particular interest in this symbiosis in recent years has led to the development of several computed artificial intelligence (AI) systems aimed at various areas of medicine. A number of studies have demonstrated that those systems allow accurate diagnoses with histological precision, thus facilitating decision-making by clinicians in real time. In the field of gastroenterology, AI has been applied in the diagnosis of pathologies of the entire digestive tract and their attached glands, and are increasingly accepted for the detection of colorectal polyps and confirming their histological classification. Studies have shown high accuracy, sensitivity, and specificity in relation to expert endoscopists, and mainly in relation to those with less experience. Other applications that are increasingly studied and with very promising results are the investigation of dysplasia in patients with Barrett's esophagus and the endoscopic and histological assessment of colon inflammation in patients with ulcerative colitis. In some cases AI is thus better than or at least equal to human abilities. However, additional studies are needed to reinforce the existing data, and mainly to determine the applicability of this technology in other indications. This review summarizes the state of the art of AI in gastroenterological pathology.

Key Words: Artificial intelligence; Computer-aided diagnosis; Deep learning; gastrointestinal endoscopy; Colorectal polyps; Dysplasia

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Core Tip: The uses of artificial intelligence (AI) in gastroenterology are growing from day to day. Many published studies have demonstrated the potential benefits of using this technology in clinical practice. We review here the most recent studies related to

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the major hot topics of AI in gastroenterology, namely colorectal polyps, dysplasia in Barrett's esophagus, and inflammation in ulcerative colitis. The machine seems to cooperate with the human, helping him to exceed his abilities and making the future of this symbiosis very promising.

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INTRODUCTION

Artificial intelligence (AI) is a concept that involves the fields of science and engineering, and was defined by one of its founders, Alan Turing, as the ability of a computer to achieve human performance in cognitive tasks[1]. Machine Learning (ML) is an AI subfield that allows the identification of patterns that can be used to analyze a particular situation[2]. ML can "learn" and improve with experience gained from provided data sets and then apply that information to similar scenarios in the future [2]. Deep learning (DL) is an AI subfield that uses artificial neural networks that are similar to the human brain, and which the capacity to learn and make decisions on its own[2]. Since 1970, AI has been used in medicine for various purposes, including improving the effectiveness of diagnosis, facilitating therapeutic and disease monitoring, and improving overall patient outcomes[2,3].

Gastroenterology is a medical specialty with great potential for integrating AI technology, as the diagnosis of gastrointestinal conditions relies much on image-based investigations (endoscopy and radiology)[4]. Numerous recent studies have investigated the applicability of AI in such diverse areas of gastroenterology, as in the investigation of dysplasia in Barrett's esophagus (BE), diagnosis of gastroesophageal reflux disease, differentiation of acute and chronic pancreatitis, detection and classification of colorectal polyps, characterization of colonic inflammatory activity in patients with inflammatory bowel disease (IBD), among others[2].

In this review, we aimed to describe the latest uses of AI in gastroenterology and the evidence that allows understanding how far these developments can supplant human abilities. We conducted a search of several platforms (PubMed, MEDLINE and EMBASE), with no time limit for articles that compared the performance of these systems and endoscopists.

AI APPLICATIONS IN DIAGNOSTIC GASTROINTESTINAL ENDOSCOPY

AI for the detection of dysplasia in Barrett's esophagus

The epidemiology of esophageal cancer has changed over the years. The increasing incidence of esophageal adenocarcinoma since the 1970s, has made it the most common type of esophageal cancer, mainly in the western countries[5,6]. BE is considered a premalignant condition[5,6] with a risk of progression to esophageal adenocarcinoma of up to 0.3% per year[7]. Endoscopic surveillance programs are based on the fact that progression tends to occur gradually from low-grade to high-grade dysplasia and esophageal adenocarcinoma. The aim is to find premalignant and malignant lesions at an early stage[8].

Because of the possible impact on the survival of the patients, there has been a growing interest in the application of AI and ML in patients with BE[9]. Several studies have reported high rates of sensitivity, specificity and accuracy with computer-aided detection (CAD) systems in detecting and delineating dysplastic lesions in patients with BE[10-13]. In comparison with expert endoscopists, the CAD system had an accuracy of 88% *vs* 73%, sensitivity of 93% *vs* 72%, and specificity of 83% *vs* 74% [10]. In addition, the area of delineation of the lesion by the CAD system overlapped that of expert endoscopists (Figure 1)[10].

Despite the promising results and the data showing that the detection speed for dysplastic lesions was compatible with the real-time use of endoscopic surveillance,

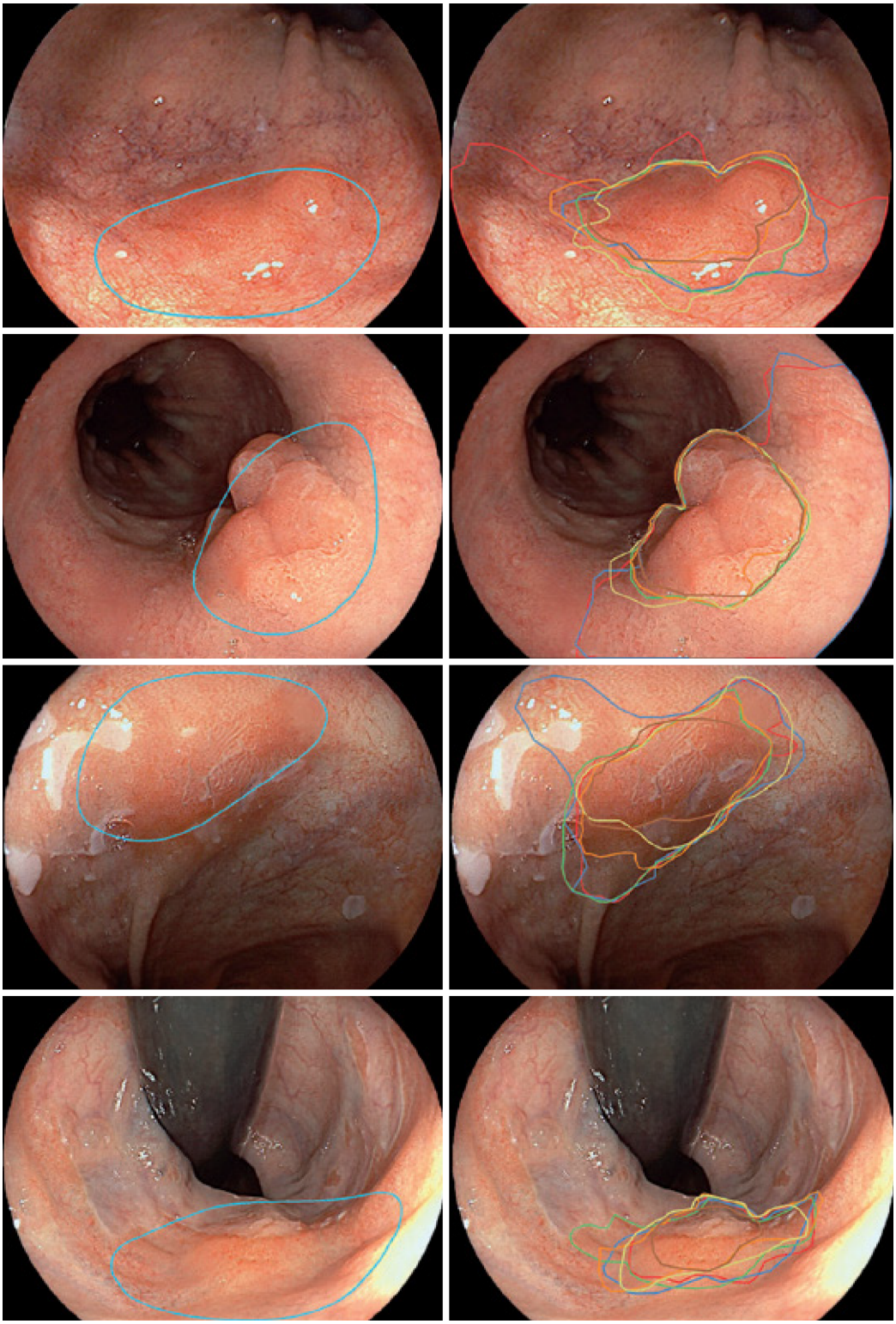


Figure 1 Delineation of Barrett's esophagus by computer-aided detection system and expert endoscopists. Images on the left are from a computer-aided detection system to identify and delineate dysplastic lesions in Barrett's esophagus with white-light imaging. Images on the right show the outlines of the same lesions made by expert endoscopists[13]. Citation: de Groof J, van der Sommen F, van der Putten J, Struyvenberg MR, Zinger S, Curvers WL, Pech O, Meining A, Neuhaus H, Bisschops R, Schoon E, With PH, Bergman JJ. The Argos project: The development of a computer-aided detection system to improve detection of Barrett's neoplasia on white-light endoscopy. *United European Gastroenterol J* 2019; 7: 538-47. Copyright © The Author(s) 2019. Published by SAGE Publications.

this application has only recently been tried in real time. Thus, Ebigbo *et al*[14] used the CAD system in 14 patients simultaneously with endoscopic examination by BE-expert endoscopists. The performance of the AI system was similar to that of the experienced endoscopists, with a sensitivity of 83.7%, a specificity of 100%, and an accuracy of 89.9%, suggesting its potential to improve endoscopic surveillance of BE,

mainly by nonexpert endoscopists.

Currently, there is no evidence that advanced imaging techniques, such as chromoendoscopy, autofluorescence endoscopy, or confocal laser endomicroscopy, offer advantages over high-definition white-light endoscopy (WLE)[7]. Volumetric laser endomicroscopy (VLE) is a recent endoscopic imaging technology that uses optical coherence tomography to produce high-resolution scans of 6-cm segments of the esophagus, with surface and subsurface image depth greater that 3 mm[15,16]. In this way, the real-time diagnosis of surface and subsurface lesions, as well as guiding their endoscopic treatment is possible[15,16]. VLE scans comprise a large amount of visual information with numerous gray-shade images that make interpretation complex and time consuming even by experts[17-19]. CAD systems have been developed that are better at identifying early BE neoplasia in *ex vivo* VLE images than VLE experts are[17], mainly when multiframe analysis is used[18]. However, further studies are needed to validate *in vivo* data. Trindade *et al*[19] also created AI image enhancement software called intelligent real-time image segmentation, that identifies three VLE features previously associated with histologic dysplasia (*i.e.* hyper-reflective surface, hyporeflective structures and lack of a layered architecture) and displays them as different colors superimposed over the VLE image to facilitate interpretation. Studies are underway to assess the effectiveness of this AI system.

AI in the detection of colorectal polyps

It is now known that colorectal cancer (CRC) surveillance with colonoscopy of both the right and left colon is associated with a reduction in CRC mortality[20-22]. The importance of a meticulous high-quality colonoscopy is a result of the inverse relationship that exists between the adenoma detection rate and the risk of interval CRC or advanced-stage CRC[23]. The high rate (up to 30%) of missed adenomas during screening colonoscopy led to the development of computer-aided detection (CADe) systems, which are programs based on DL, in an attempt to mitigate this problem[24]. The first study[25] on the use of CADe in the detection of colon polyps was published in 2003, and it demonstrated good results. However, only static images were used. Since then, several systems have been developed and used with real-time endoscopy. In recent systematic review and meta-analysis, Hassan *et al*[26] assessed the relationship between the increased detection of polyps and the main features of the detected lesions. The review included five randomized clinical trials with a total of 4354 patients (2163 in the CADe and 2191 in the control group). The highlight of the results was that the adenoma detection rate in the CADe group was significantly higher (36.6%) than that in the control group (25.2%) regardless of adenoma size, location, and morphology of the polyps. The rates of detection of advanced adenomas were not significantly different. The authors concluded that AI could benefit colorectal cancer surveillance, as the factors that affect the detection of lesions by human observers, such as size and morphology, do not interfere with detection by the AI system. In addition, the study shows that the AI system did not affect the efficiency of colonoscopy, maintaining similar withdrawal time in both groups. The results are supported by those in prospective study by Liu *et al*[27] in which the adenoma detection rate was higher in the CADe group than in the control group (29.01% *vs* 20.91%) and a meta-analysis by Barua *et al*[28] that found an absolute increase of 10.3% in the detection rate of adenomas. The increase was mainly the result of increased detection of nonadvanced diminutive adenomas, serrated adenomas, and hyperplastic polyps[26,27]. In addition to the smaller size, other characteristics of the polyps first detected by the CADe system were isochromia, flat shape, unclear boundary, partly behind colon folds, and at the edge of the visual field[27]. Thus, the features of the polyps seem to contribute to a higher probability of missed lesions, however Lui *et al*[29] found that 79% of missed lesions can be detected with AI support during the first examination and considered that the main cause of missed lesions was human error, such as less endoscopist experience or the presence of multiple polyps that can increase endoscopist distraction or fatigue. We did not find consistent evidence for the use of advanced imaging techniques in the detection of polyps by CAD systems.

AI in the histological characterization of colorectal polyps

One of the problems of CRC surveillance is the high cost of polypectomy and pathological examination, in particular given the high prevalence of diminutive polyps ≤ 5 mm[30]. It has been proven that a resect and discard strategy for diminutive polyps has an important economic impact, without losing efficacy in CRC surveillance[30]. An optical biopsy of diminutive polyps supported by image-enhanced endoscopic may be appropriate. The Preservation and Incorporation of Valuable endoscopic Innovations (PIVI) group of the American Society for Gastrointestinal Endoscopy (ASGE)[31]

recommends that for a "diagnose-and-leave" strategy, endoscopic diagnosis should provide a 90% or higher negative predictive value for adenomatous histology when used with high confidence. For a "resect and discard" strategy for adenomas ≤ 5 mm, it is recommended that an optical biopsy with high confidence, when combined with a histologic assessment of polyps larger than 5 mm, should provide ≥ 90% agreement in the assignment of post polypectomy surveillance intervals compared with decisions based on pathological assessment of all identified polyps.

The optical diagnosis of colorectal polyps has not yet been widely implemented because of the lack of endoscopist experience and the considerable learning curve of advanced imaging modalities[32,33]. To overcome this problem, AI systems such as, computer-aided diagnostic (CADx) that help to predict the histology (*i.e.* neoplastic or non-neoplastic) of detected polyps (Figure 2) have been developed with the aim of avoiding unnecessary endoscopic biopsies or resection[33-35]. Yang *et al*[35] created DL models that classify colorectal lesions histologically using white-light colonoscopy images. They found an accuracy of 79.5% in distinguishing neoplastic and non-neoplastic lesions and 87.1% in distinguishing advanced and nonadvanced colorectal lesions. When comparing the performance of endoscopists and CADx for the classification of polyps, they found that CADx performed better than less experienced endoscopists, but not better than experts.

Combinations of advanced imaging modalities, such as narrow-band imaging (NBI) or endocytoscopy, and CAD systems improved the performance of AI-assisted optical biopsy[34]. Several studies have shown the potential of combining NBI with CADx in the classification of polyps[36-38]. Song *et al*[37] developed a DL model to predict colorectal polyp histology based on NBI near-focus images. Their study showed a good diagnostic accuracy (81.3%-82.4%) that was comparable to expert endoscopists and was significantly more accurate than trainee endoscopists. CADx performance was consistent regardless of polyp size, location, or morphology, with an average histological assessment time of 0.02-0.04 s. They concluded that an AI CAD system can help inexperienced endoscopists in the histological assessment of colorectal polyps and increase the confidence of experts regarding their assessments.

A recent systematic review and meta-analysis that included 18 studies (15 retrospective and three prospective) and 7680 images of colorectal polyps was performed to evaluate the prediction of histology[39]. AI had an accuracy of 0.96 (AUC), with a sensitivity of 92.3% and a specificity of 89.8%. Comparing studies that used NBI with those that used non-NBI techniques, they found a significantly better accuracy (AUC 0.98 *vs* 0.84) in the NBI studies. When they evaluated performance in the histological characterization of diminutive polyps, they found that the accuracy overlapped and that the pooled negative predictive value was 91.3%-95.1%, which complies with the threshold of the "resect and discard" strategy of the ASGE. The difference in the performance of AI and endoscopists was not significant except in studies that included nonexpert endoscopists, in which the performance AI was significantly better than that of nonexpert endoscopists.

Endocytoscopy (EC) is an advanced endoscopic technique in which a contact light microscopy system is integrated into the distal tip of a conventional colonoscope[40]. This technique has performed well in differentiating between diminutive neoplastic and non-neoplastic colorectal polyps both alone[41] and in combination with NBI (EC-NBI)[42]. The combined use of EC and CAD (EC-CAD) to classify the histology of colorectal polyps has been studied. According to Mori *et al*[40], this technology achieved a sensitivity of 92%, a specificity of 79.5%, and an accuracy of 89.2%. The performance was comparable to EC image evaluation by experts, but significantly better than evaluation by trainees for identifying neoplastic polyps.

AI in IBD

Ulcerative colitis (UC) is a chronic inflammatory disease, and the therapeutic goals have changed over the last decades, from the treatment of symptoms toward mucosal healing, with the aim of modifying the natural history of disease[43-45]. Mucosal healing can be defined by endoscopic evaluation because the inflammation is limited to the mucosal layer[44]. However, it is currently known that histological evidence of inflammation, even in patients with mucosal healing, is a prognostic marker of a two-to-three-fold increase in the risk of colitis relapse and colon cancer[44,45]. Consequently, assessment of inflammatory activity and severity are important for the choice of therapeutic and follow-up strategies. This supports performing colonoscopies with biopsies and visually interpretation of mucosal and histologic parameters of inflammation using standardized disease activity measures[46]. Visual interpretation is subject to bias, interobserver, and intraobserver variability. Histological evaluation requires time for processing and interpretation, which limits the ability to make

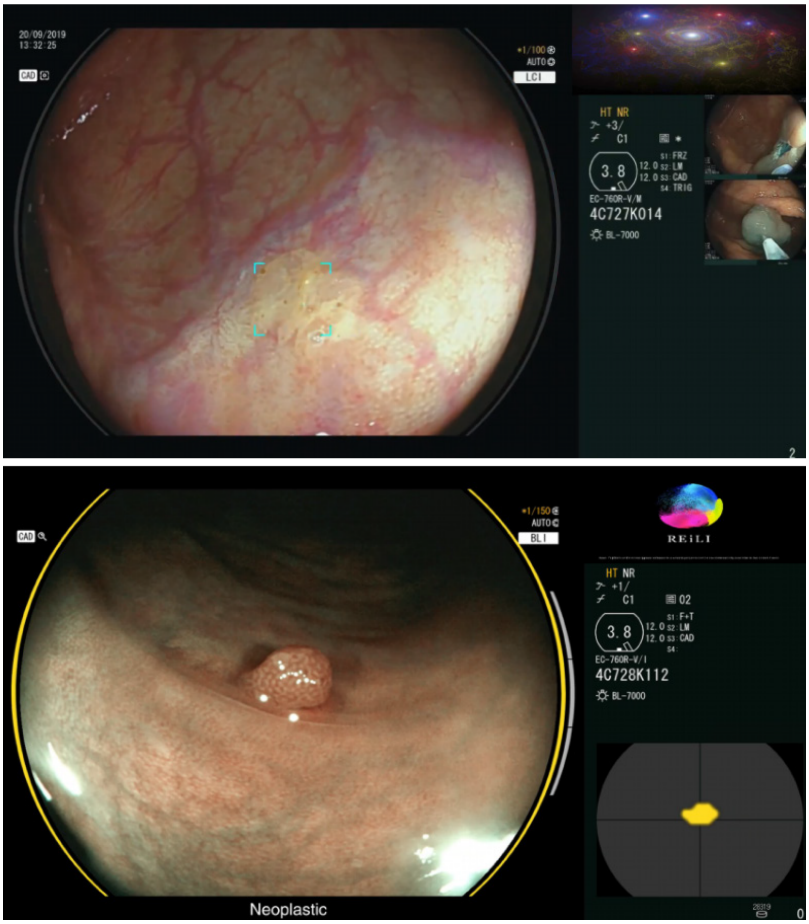


Figure 2 Computer-aided detection system for detecting and classifying colorectal polyps. The top image is a polypoid lesion (delimited by blue marks) identified by a CADe system. The bottom image is a lesion classified as neoplastic by the CADx system with a high degree of confidence (three gray bars on the right side of the image)[33]. Citation: Mori Y, Neumann H, Misawa M, Kudo SE, Bretthauer M. Artificial intelligence in colonoscopy - Now on the market. What's next? *J Gastroenterol Hepatol* 2021; 36(1): 7-11. Copyright © The Author(s) 2020. Published by Journal of Gastroenterology and Hepatology Foundation and John Wiley & Sons Australia, Ltd.

decisions in real time[46].

Several studies have used AI for the macroscopic and histological characterization of colic mucosa in UC. Takenaka *et al*[47] developed a deep neural network for evaluation of UC with the aim of predicting endoscopic remission, a UC endoscopic index of severity score of 0; and histologic remission, a Geboes score of ≤ 3 points using endoscopic images. In the training set, they used 40,758 images of colonoscopies and the histologic results of 6885 biopsies from the same patients. They validated the results in a prospective study of 875 patients with 4187 endoscopic images and 4104 biopsy specimens. In the validation phase, the AI system was able to identify patients with endoscopic remission with 90.1% of accuracy and histologic remission with 92.9% of accuracy using only endoscopic images. The results suggest that computer-aided diagnosis can reliably assist endoscopic and histologic disease activity in UC without the need for mucosal biopsies. It should be noted that the study used only white-light images (WLIs). Chromoendoscopy was not used after WLI evaluation.

However, there are doubts that conventional WLI can reliably identify persistent histologic inflammation. Alternatives such as NBI, confocal laser endomicroscopy, or EC have emerged[48]. Despite their excellent diagnostic performance, these techniques are highly dependent on experienced endoscopists[48]. Maeda *et al*[48] developed a CAD system that used AI and EC to predict persistent histologic inflammation (Figure 3). It was used to retrospectively evaluate 12900 EC images for ML and 9935 EC images for validation, in addition assessment of biopsy samples by experienced pathologists blinded to the endoscopy results. The algorithm had 74% sensitivity, 97% specificity, and 91% accuracy in identifying histologic inflammation, and perfect reproducibility. The results are promising, and the authors concluded that the symbiosis achieved with the CAD system and EC has the potential to support immediate intervention and ultimately reduce the number of required biopsy samples.

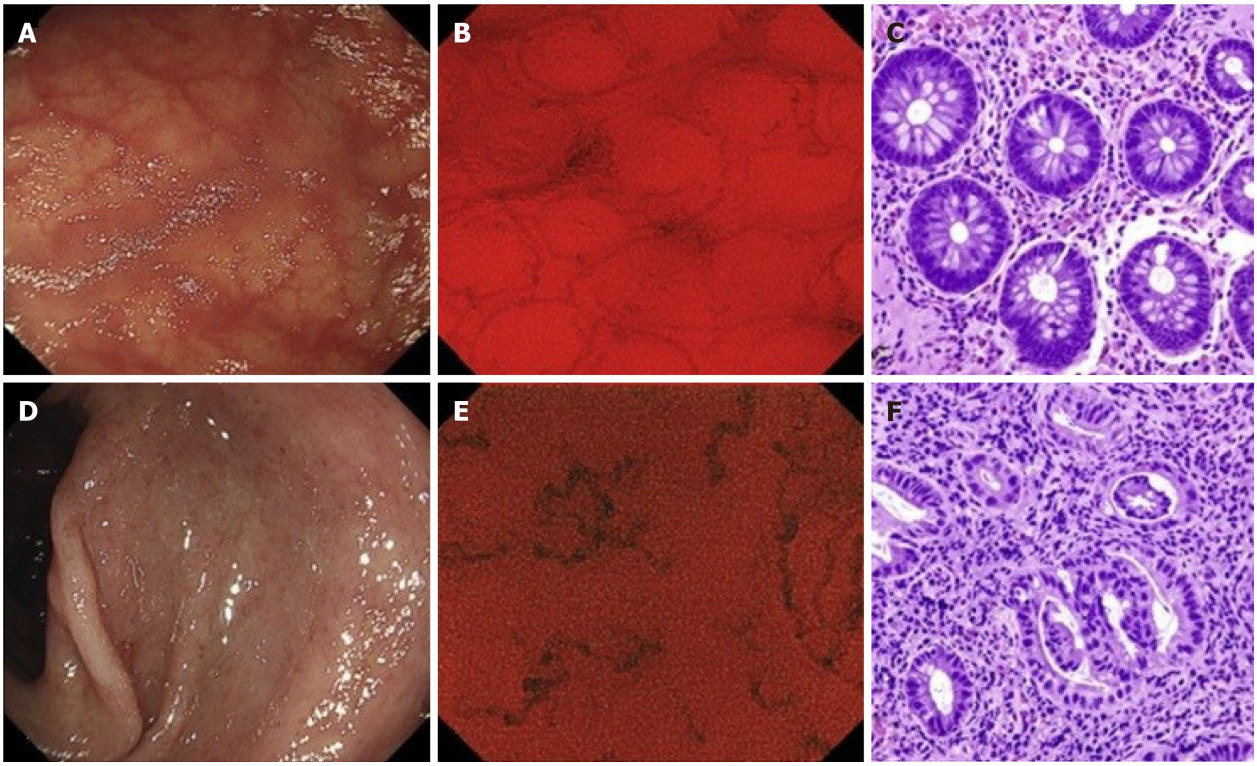


Figure 3 Application of computer-aided detection systems to endocytoscopy. A, D: Conventional endoscopic images in WLI; B, E: Endocytoscopic images; C, F: Histological images. A CAD system evaluated the endocytoscopy image B: "healing" and E: active inflammation. The classifications were later confirmed by the pathologist[48]. Citation: Maeda Y, Kudo SE, Mori Y, Misawa M, Ogata N, Sasanuma S, Wakamura K, Oda M, Mori K, Ohtsuka K. Fully automated diagnostic system with artificial intelligence using endocytoscopy to identify the presence of histologic inflammation associated with ulcerative colitis (with video). *Gastrointest Endosc* 2019; 89(2): 408-415. Copyright © The Author(s) 2019 by the American Society for Gastrointestinal Endoscopy. Published by Elsevier, Inc.

There is a lack of published studies of the potential benefit of AI in assessment of disease activity in Crohn's disease. Evidence of the applicability of AI to the surveillance of dysplasia in patients with IBD is still lacking. Maeda *et al*[49] described a case in which an AI system assisted in the detection of dysplastic lesions in a patient with UC. They concluded that AI may have potential in this context, especially for nonexpert endoscopists.

CONCLUSION

The evidence for a role of AI in diagnostic endoscopy is growing, with increasing supporting evidence in dysplasia detection in BE, identification and assessment of colorectal polyps, and characterization of colonic mucosa in UC. A number of possible applications are still under evaluation (Table 1)[50-58]. *Deus ex machina* is a Latin expression meaning "God from the machine". The term was coined from the conventions of ancient Greek theatre, where actors who were playing gods were brought onto stage using a machine[59]. Later it was used in the story plots of theatre plays and movies to describe un unexpected and sudden solution to resolve main issues or tragedies. AI is currently being developed for use in endoscopy daily practice. It seems to be a quick solution of the long-standing problems of the interobserver agreement and the subjective "feeling" and accuracy of the endoscopist in diagnostic exams. Apart from the complexity of improving or overcoming human evaluation, there are also availability issues that will limit the access to this new technology.

In the meantime, the added value of electronic systems to human practice must not be underrated as they would definitely contribute to the standardization of diagnostic endoscopy worldwide. Apart from that, savings in the time, effort, economic, and human resources needed for diagnostic exams, would make room for improvements in endoscopic therapeutic procedures, which are growing in complexity over time.

In conclusion, AI is currently used to optimize diagnostic endoscopy procedures, with growing indications and evidence over time. Although it may not be ready for

Table 1 Potential applications of artificial intelligence in gastrointestinal pathology (under evaluation)

Disease	Detection	Ref.
Esophageal squamous cell carcinoma (ESCC)	Detection of early ESCC	Yang *et al*[50]
		Kumagai *et al*[51]
Helicobacter pylori infection	Diagnosis of *Helicobacter pylori* infection	Mohan *et al*[52]
Gastric cancer (GC)	Detection of preneoplastic lesions and early gastric cancerPrediction the invasion depth of GC	Anta *et al*[53]
		Nagao *et al*[54]
Celiac disease	Early detection of celiac disease	Sana *et al*[55]
Gastrointestinal bleeding	Detection of small bowel bleeding with wireless capsule endoscopic	Aoki *et al*[56]
		Tsuboi *et al*[57]
Pancreatic lesions	Differentiation of pancreatic cancer from chronic pancreatitis and normal pancreas	Tonozuka *et al* [58]

prime-time use worldwide, the future of AI is near. It will depend on us, human endoscopists, to define how far these machines can help us with (and solve?) our naturally imperfect issues.

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MINIREVIEWS

Faecal microbiota transplantation enhances efficacy of immune checkpoint inhibitors therapy against cancer

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Abstract

Even though immune checkpoint inhibitors (ICIs) are effective on multiple cancer types, there are still many non-responding patients. A possible factor put forward that may influence the efficacy of ICIs is the gut microbiota. Additionally, faecal microbiota transplantation may enhance efficacy of ICIs. Nevertheless, the data available in this field are insufficient, and relevant scientific work has just commenced. As a result, the current work reviewed the latest research on the association of gut microbiota with ICI treatments based on anti-programmed cell death protein 1 antibody and anti- cytotoxic T-lymphocyte-associated protein 4 antibody and explored the therapeutic potential of faecal microbiota transplantation in combination with ICI therapy in the future.

Key Words: Gut microbiome; Immunotherapy; Programmed cell death protein 1/programmed cell death protein ligand 1; Cytotoxic T-lymphocyte-associated protein 4; Immune checkpoint inhibitors resistance; Faecal microbiota transplantation

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Core Tip: Gut microbiota composition is closely associated with the efficacy of immune checkpoint inhibitors (ICIs). Specific species among the intestinal commensal bacteria may play a key role in the efficacy of ICIs against cancer. Faecal microbiota transplantation may enhance efficacy of ICIs.

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INTRODUCTION

Immune checkpoint molecules can modulate the immune system in the host *via* the transduction of immunosuppressive co-signals into the immunocompetent cells[1-5]. Typically, Programmed cell death protein 1 (PD-1)/programmed cell death protein ligand 1 (PD-L1) (CD274), PD-L2 (CD273), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, CD152) are the most well-known examples[6-13]. These molecules are expressed in suitable cells at the suitable timing to exert their vital parts in the prevention of over-activated immune system in the host and the maintenance of immunological tolerance and homeostasis[1,2,5]. At the same time, immune checkpoint molecules show abnormal expression within tumour tissues[3,14-16]. Therefore, a strong immunosuppressive environment will be produced within tumour tissues, leading to resistance to treatment of numerous cancers. Immune checkpoint inhibitors (ICIs) mainly function to alleviate or destroy the immunosuppression mechanisms involved in tumour microenvironment (TME) by the use of inhibitory agents targeting the immune checkpoint molecules[2,5,17]. At present, anti-CTLA-4 (like ipilimumab), anti-PD-1 (such as pembrolizumab, nivolumab, and anti-PD-L1 (such as atezolizumab, durvalumab, avelumab) antibodies have been applied in treating several cancers in the word[18-23].

At present, checkpoint blockade still shows high effectiveness on certain cases, but just about 10%-30% cancers can achieve treatment responses. The combined used of ICIs is associated with a higher response rate and greater toxicity[24], regardless of the limited research on the ICI treatment. There are several ICI resistance mechanisms related to the low response rate, which are low PD-L1 expression, low tumour mutational burden, local immunosuppression, weak tumuor cell antigenicity, tumour-infiltrating lymphocytes (TILs) functional exhaustion, no priming, and defected antigen presentation in the process of priming[25].

In addition, gut microbiome is suggested to be the potential factor that determines ICI efficacy. There are more than 100 trillion bacteria in the human gut, among which 500-1000 bacterial species have been identified to affect the mucosal immune system and exert vital parts in immune system operation under the normal or disease state [26]. Intestinal symbiotic bacteria may exert inflammatory or beneficial function while interacting with host immune system in intestinal lymphoid tissues. Therefore, faecal microbiota transplantation (FMT) can potentially improve the ICI efficacy. Nonetheless, there is only limited information on this topic, and related scientific work is merely at the beginning stage. The emergence of novel techniques has made it possible to investigate systemically the gut microbiota, which also sheds more light on the gut microbial compositions and their pathological variance. The present work aimed to review the latest research on the associations of gut microbiota with immune systems and ICI treatments based on anti-PD-1 antibody (Ab) and anti-CTLA-4 Ab and to explore the therapeutic potential of FMT combined with ICI therapy in the future.

ICIS

Two steps are necessary to activate tumour-specific T cells. Firstly, the selective binding of T cell receptor (TCR) to major histocompatibility complex I that has antigen-anchoring peptides[27]. Secondly, further amplification of the activation signal of TCR/CD3 complex is performed after the synergistic effect with co-stimulatory signals like OX40, CD28, and inducible T cell co-stimulator, which finally results in T cell priming and activation[27]. By contrast, co-inhibitory signals (also known as the immune checkpoints), including PD-1, CTLA-4, T cell immunoglobulin domain, mucin domain-3, and lymphocyte activation gene-3, inhibit T cell activation *via* offsetting CD28- or TCR/CD3-mediated tyrosine phosphorylation through the intracellular immunoreceptor tyrosine–based inhibition motif[28-30]. Tumour cells are likely to enhance the co-inhibitory signalling pathway activity for the sake of immune escape [31,32]. ICIs can decrease the tumour antigen immune tolerance and restore the anticancer response. Anti-CTLA-4 and anti-PD-1/PD-L1 are used to treat several cancers[33-38]. Nevertheless, there is a great potential to enhance the anticancer effect of ICI.

EFFECT OF GUT MICROBIOME ON THE EFFICACY OF ICIS

It has been recognized that gut microbiome is involved in cancer genesis and the immune surveillance that suppresses tumour progression[39-42]. Certain commensals may display the synergetic effects with treatments such as surgery, chemotherapy, radiotherapy, and immunotherapy after affecting the immune homeostasis in the intestine and immune adjustment of secondary immune organs[43-52]. ICIs can regulate tumour regression through enhancing the immune activation in the host. A series of studies suggested that gut microbiota composition shows close association with the efficacy of ICIs (Tables 1 and 2). At the same time, we revealed the potential mechanisms by which gut microbiome may be involved in the ICI efficacy (Figures 1 and 2).

Effect of gut microbiota on anti-PD-1/PD-L1 therapy

The PD-1/PD-L1 blockage treatment blocks the negative signals transduced by the PD-1 intracellular domains (like immunoreceptor tyrosine–based inhibition motif, immunoreceptor tyrosine–based switch motif)[53]. Typically, PD-1/PD-L1 blockage has been identified to promote T cell activation resulting from CD28 and TCR/CD3 while promoting T cell growth and survival by the activation of Ras-Raf-mitogen activated protein kinase and phosphatidylinositol 3 kinase-AKT signalling[54,55]. The PD-1/PD-L1 blockage treatment has been approved to treat certain malignant tumours, including non-small cell lung cancer (NSCLC), colorectal cancer (CRC), kidney cell cancer, and melanoma[56,57]. Biomarkers that contain the TIL status, PD-L1 expression, or deficiency of the mismatch repair system are tightly associated with the efficacy of PD-1/PD-L1 blockage treatment[58]. Besides those above-mentioned factors, gut microbiota contributes to difference in treatment responses as well[59].

In 2015, some investigators discover the relationship of gut microbiota with the efficacy of anti-PD-1 therapy using a mouse model[60]. Sivan *et al*[60] explored the therapeutic effect of anti-PD-1 therapy on C57BL/6 mice with genetic similarity, mice bearing the subcutaneous B16. SIY melanoma were obtained from two distinct mouse facilities [namely, Taconic Farms (TAC) and Jackson Laboratory (JAX)], which had markedly heterogeneous gut microbial compositions[60]. As a result, among the JAX populations, tumour growth was slower with higher sensitivity to the PD-1 blockage treatment. Such difference might be associated with the immune response. To be specific, JAX mice showed markedly enhanced $CD8^+$ T cell aggregation within the tumour and tumour-specific T cell responses compared with the TAC counterparts. Further study suggested that the difference was abrogated by cohousing. In addition, when faecal microbiome was transferred from JAX to TAC, specific TILs increased and tumour development was suppressed. It was interesting that, in TAC, just the faecal microbiome transferred from JAX was able to suppress tumour development in the same degree with PD-1 blockage therapy, and it had synergistically regressed tumour development with PD-1 blockade therapy[60]. Gut microbiome analysis demonstrated that the abundance of *Bifidobacterium* was markedly increased in JAX. Meanwhile, the abundance of *Bifidobacterium* was significantly related to tumour specific immune cytotoxicity[60]. Administrating the commercial Bifidobacterium cocktail (namely, *Bifidobacterium longum* and *Bifidobacterium breve*) significantly suppressed tumour growth, particularly when it was used in combination with the PD-1 blockage treatment[60]. It was suggested that such increased anticancer activity was associated with the higher interferon (IFN)-γ production, greater tumour-specific $CD8^+$ T cell proportion, and alterations of dendritic cell (DC) functions[60].

Xu *et al*[61] investigated the roles of gut microbiome within the MSS-type mice bearing CRC that received diverse antibiotic treatments in the response to PD-1 Ab therapy. Following PD-1 Ab therapy, injecting antibiotics offset the therapeutic effect of PD-1 Ab on suppressing tumour development relative to control group[61]. Besides, control group showed enrichment of *Bacteroidales*_S24-7 and *Bacteroides_sp*._CAG:927. At the same time, mice receiving colistin treatment showed enrichment of Bacteroides_sp._CAG:927, *Bacteroides* and *Prevotella_sp*._CAG: 1031, whereas mice receiving vancomycin treatment showed enrichment of *Akkermansia_muciniphila* and *Prevotella_sp*._CAG:485. For mice receiving vancomycin treatment, most metabolites were associated with the glycerophospholipid metabolic pathway, confirming to the metagenomic prediction pathway. Additionally, *Akkermansia* and *Prevotella_sp*._CAG:485 contributed to maintaining the therapeutic effect of PD-1 Ab through impacting glycerophospholipid metabolism[61]. Gut microbial alteration resulted in alterations of the glycerophospholipid metabolism degree, thereby affecting immune cytokine expression [such as interleukin (IL)-2 and IFN-γ] within TME, giving rise to the diverse PD-1 Ab efficacy[61]. The above results reveal that gut microbial alter-

Table 1 Changes in microbiota composition associated with anti-programmed cell death protein 1/programmed cell death protein ligand 1 treatment efficacy against cancer and potential strategies for improving efficacy

Models	Disease	Implicated microbiota	New strategies	Implicated microbiota	Ref.
Mice	Melanoma	*Bifidobacterium*↑	(1) FMT; and (2) Commercial cocktail of *Bifidobacterium* including *Bifidobacterium breve* and *B. longum*	NO	Sivan *et al*[60], 2015
Mice	CRC	*Bacteroides_sp._*CAG:927↑, *Bacteroidales_*S24-7↑, *Akkermansia muciniphila*↑	NO	NO	Xu *et al*[61], 2020
Mice	CT26 tumours	NO	GQD	*s__Bacteroides acidifaciens*↑, *s__uncultured_organism_g__norank_f__Bacteroidales_*S24-7↑	Lv *et al*[62], 2019
Mice	RCC	NO	(1) FMT; (2) *A. muciniphila*; and (3) *Bacteroides salyersiae*	NO	Derosa *et al*[66], 2020
Mice	Melanoma	NO	FMT	NO	Matson *et al*[68], 2018
Mice	MCA-205 sarcoma	NO	(1) FMT; (2) *A. muciniphila*; and (3) *A. muciniphila* with *Enterococcus hirae*; *Alistipes indistinctus*	NO	Routy *et al*[69], 2018
Mice	(1) RET; and (2) Melanoma	NO	(1) *A. muciniphila*; and (2) *A. muciniphila* with *Enterococcus hirae*;	NO	Routy *et al*[69], 2018
Human	NSCLC	*Parabacteroides*↑, *Methanobrevibacter*↑, *Veillonella*↓, *Selenomonadales*↓, *Negativicutes*↓	NO	NO	Song *et al*[63], 2020
Human	NSCLC	Gut microbial diversity↑, *Alistipes putredinis*↑, *B. longum*↑, *Prevotella copri*↑, *Ruminococcus unclassified*↓	NO	NO	Jin *et al*[64], 2019
Human	NSCLC	Altered gut microbiota metabolome	NO	NO	Botticelli *et al*[65], 2020
Human	RCC	*A. muciniphila* ↑, *Bacteroides salyersiae*↑, *Clostridium hathewayi*↓	NO	NO	Derosa *et al*[66], 2020
Human	Melanoma	Gut microbial diversity↑, *Clostridiales/Ruminococcaceae*↑, *Faecalibacterium*↑, *Anaerotruncus colihominis*↓, *Bacteroides thetaiotaomicron*↓, *Escherichia coli*↓	NO	NO	Gopalakrishnan *et al*[67], 2018
Human	Melanoma	*Bifidobacterium adolescentis*↑, *B. longum*↑, *Collinsella aerofaciens*↑, *Enterococcus faecium*↑, *Klebsiella pneumoniae*↑, *Lactobacillus species*↑, *Parabacteroides merdae*↑, *Veillonella parvula*↑, *Ruminococcus obeum*↓, *Roseburia intestinalis*↓	NO	NO	Matson *et al*[68], 2018
Human	NSCLC and RCC	*A. muciniphila* ↑	NO	NO	Routy *et al*[69], 2018
Human	Melanoma	NO	FMT	NO	Baruch *et al*[83], 2021

NO: No test or no research; FMT: Faecal microbiota transplantation; CRC: Colorectal cancer; RCC: Renal cell carcinoma; NSCLC: Non-small cell lung cancer; GQD: Gegen Qinlian decoction.

ations have certain impacts on the glycerophospholipid metabolic pathway, thus modulating the efficacy of PD-1 Ab immunotherapy in treating MSS-type mice bearing CRC.

Table 2 Changes in microbiota composition associated with anti-cytotoxic T-lymphocyte-associated protein 4 efficacy treatment against cancer and potential strategies for improving efficacy

Models	Disease	Implicated microbiota	New strategies	Implicated microbiota	Ref.
Mice	MCA205 sarcomas	*Clostridiales*↑, *Bacteroides thetaiotaomicron*↑, *B. uniformis*↑, *Bacteroidales*↓, *Burkholderiales*↓,	(1) *B. thetaiotaomicron*; (2) *B fragilis*; and (3) *Burkholderia cepacia*	NO	Vétizou *et al*[70], 2015
Mice	CRC	NO	*Lactobacillus acidophilus* cell lysates	NO	Zhuo *et al* [73], 2019
Mice	CRC	NO	(1) *Bifidobacterium pseudolongum*; (2) *Lactobacillus johnsonii*; (3) *Olsenella spp*; *and* (4) Metabolite inosine	NO	Mager *et al* [74], 2020
Huamn	Melanoma	*Faecalibacterium genus*↑, unclassified *Ruminococcus*↑, *Lachnospiraceae genus*↑, *Clostridium* XIVa↑, *Blautia*↑, Butyrate producing bacterium↑, *Gemmiger formicilis*↑, *Bacteroides*↓, *B. fragilis*↓, *B. thetaiotaomicron*↓	NO	NO	Chaput *et al*[72], 2017

NO: No test or no research; CRC: Colorectal cancer.

Recently, Lv *et al*[62] discovered that when Gegen Qinlian decoction (GQD) (one of the representative traditional Chinese medicine prescriptions) was used in conjunction with the anti-mouse PD-1 therapy in the xenograft model, it had potent effect on suppressing CT26 tumour growth. Besides, analysis on the gut microbiota also suggested that GQD used in combination with anti-mouse PD-1 therapy markedly enriched *s__uncultured_organism _g__norank_f__Bacteroidales_ S24-7_*and *s__Bacteroides_acidifaciens* group[62]. As indicated by metabolomic analysis results, metabolites with profound changes were detected in the combined treatment group [62]. Furthermore, the sphingolipid metabolism and glycerophospholipid metabolism metabolic pathways were examined[62]. Particularly, GQD combined with anti-mouse PD-1 treatment markedly promoted the fraction of $CD8^+$ T cell subset within tumour tissue and peripheral blood samples and up-regulated IFN-γ level (an important factor of the anticancer immunotherapy)[62]. Moreover, GQD combined with anti-mouse PD-1 treatment decreased PD-1 expression while increasing IL-2 expression, revealing that such combined treatment suppressed the inhibitory checkpoints to restore efficiently T-cell functions[62]. Taken together, such findings revealed that GQD remodels gut microbiota to promote the anti-CRC efficacy of PD-1 blockade, and microsatellite stability was achieved.

Inspired by these results obtained from mouse models, many articles have been conducted to examine the association of gut microbiota with anti-PD-1 therapy among cancer cases. Song *et al*[63] explored the association of gut microbial structure and metabolomic features in the context of NSCLC with the anti-PD-1 therapy efficacy. According to analysis results of gut microbiome, cases from progression-free survival (PFS) ≥ 6-mo group showed markedly increased β-diversity within gut microbiota relative to that of PFS < 6-mo group[63]. Besides, those from PFS ≥ 6-mo group showed enrichment of *Methanobrevibacter* and *Parabacteroides*, whereas those from PFS < 6-mo group showed enrichment of *Selenomonadales*, *Negativicutes*, and *Veillonella*[63]. Furthermore, the protein families of function groups were studied using the COG, CAZy, and KO databases. As a result, 264, 859, and 390 functional groups were enriched in the above three databases, respectively, and significant differences were detected between the two groups. As revealed by analysis on bacterial metabolites, differences in the metabolic potentials of methane and methanol were significant between the two groups[63].

Jin *et al*[64] examined the association of gut microbiome with the clinical outcomes among the Chinese NSCLC cases receiving the anti–PD-1 therapy. Thereafter, patients were grouped as non-responder and responder groups based on the clinical response evaluated by the Response Evaluation Criteria in Solid Tumor version 1.1[64]. As a result, responders showed a greater gut microbial diversity at the beginning and stable composition in the process of treatment[64]. Besides, those showing higher microbial diversity were associated with the remarkably longer PFS in comparison with patients showing a lower diversity[64]. Differences in composition were detected between both groups, among which, *Alistipes putredinis*, *Prevotella copri*, and *Bifidobacterium longum* were enriched in responder group, while *Ruminococcus unclassified* was enriched in non-responder group[64]. In addition, the author applied multicolor flow cytometry to

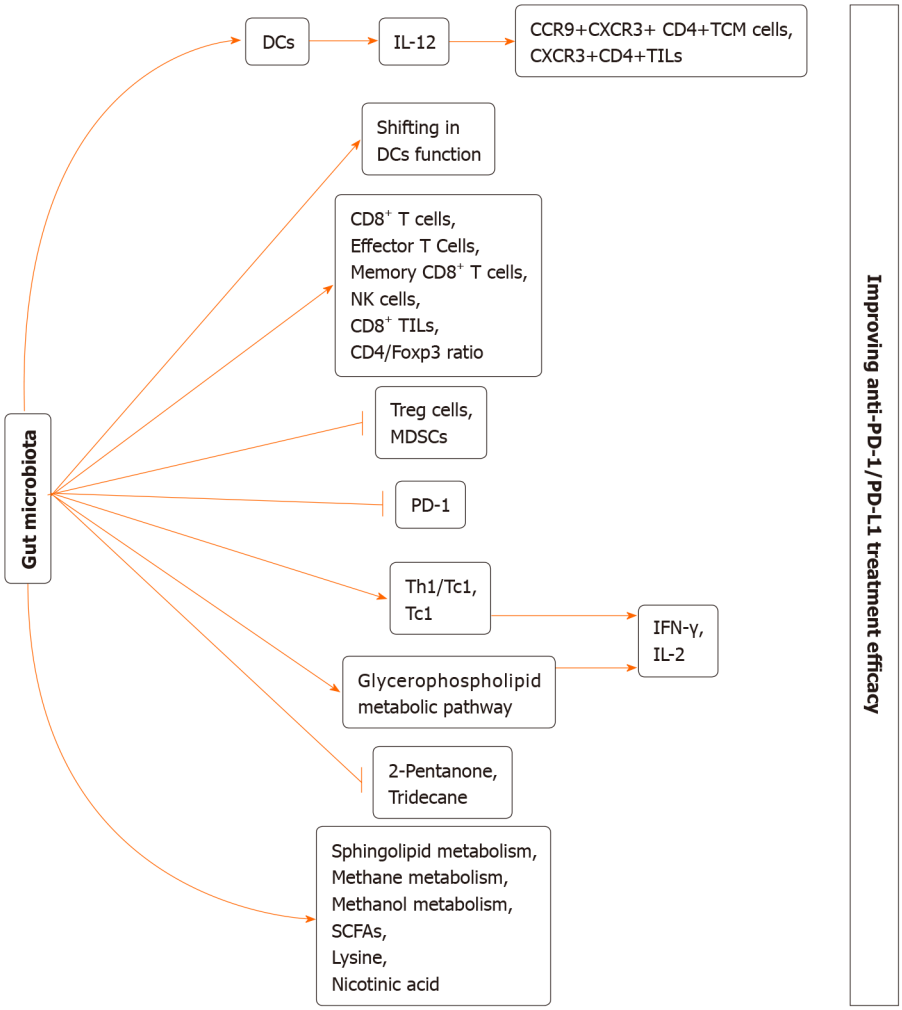


Figure 1 The potential mechanism of gut microbiome regulating anti-programmed cell death protein 1/programmed cell death protein ligand 1 treatment efficacy. (1) Gut microbiota may increase interleukin (IL)-12 production by dendritic cells (DCs), thereby increasing the CCR9$^+$CXCR3$^+$CD4$^+$ central memory T cells and CXCR3$^+$CD4$^+$ tumour-infiltrating lymphocytes (TILs); (2) Gut microbiota may shift in the function of DCs; (3) Gut microbiota may increase CD8$^+$ T cells, effector T Cells, memory CD8$^+$ T cells, natural killer cells, CD8$^+$ TILs, and CD4/Foxp3 ratio; (4) Gut microbiota may decrease numbers of regulatory T cells and myeloid-derived suppressor cells; gut microbiota may downregulate programmed cell death protein 1 expression; (5) Gut microbiota may induce Th1/Tc1and Tc1 immune response, thereby increasing the interferon-γ and IL-2 secretion; (6) Gut microbiota may enhance glycerophospholipid metabolic pathway, thereby increasing the interferon-γ and IL-2 secretion; (7) Gut microbiota may decrease 2-pentanone and tridecane production; and (8) Gut microbiota may increase sphingolipid metabolism, methane metabolism, methanol metabolism, short chain fatty acids production, lysine production and nicotinic acid production. Altogether, all of these approaches may eventually improve anti-programmed cell death protein 1-1/programmed cell death protein ligand 1 treatment efficacy. DCs: Dendritic cells; T_{CM}: Central memory T; Treg: Regulatory T; MDSCs: Myeloid-derived suppressor cells; SCFAs: Short chain fatty acids; PD-1: Programmed cell death protein 1; IFN-γ: Interferon.

analyzed the systemic immune responses, which suggested that patients showing a greater gut microbial diversity were associated with higher proportions of peripheral blood natural killer cell and unique memory CD8$^+$ T cell subsets upon anti–PD-1 treatment[64]. Botticelli and coworkers[65] also investigated the impact of gut microbial metabolome on anti-PD-1 therapy efficacy among NSCLC cases. As a result, 36% cases presented early progression, whereas the rest 64% showed progression at 12 mo later[65]. Besides, as revealed by gut microbiota metabolomic profiling, tridecane (alkane) and 2-Pentanone (ketone) were tightly related to early progression; by contrast, nicotinic acid, lysine and short chain fatty acids (namely, butyrate, propionate) were closely related to long-term benefits[65].

Recently, Derosa *et al*[66] assessed the significance of faecal bacterial composition in the anti-PD-1 treatment effect among patients with advanced renal cell carcinoma (RCC). Relative to RCC cases who received PD-1 blockage treatment with no use of antibiotics, RCC cases who received anti-PD-1 treatment in the presence of antibiotic treatment had evidently decreased objective response rates, which remarkably impacted the microbial composition. As a result, certain species like *Clostridium hathewayi* were dominant, and their abundances were higher in faecal samples of RCC cases relative to normal subjects. Tyrosine kinase inhibitors administered before

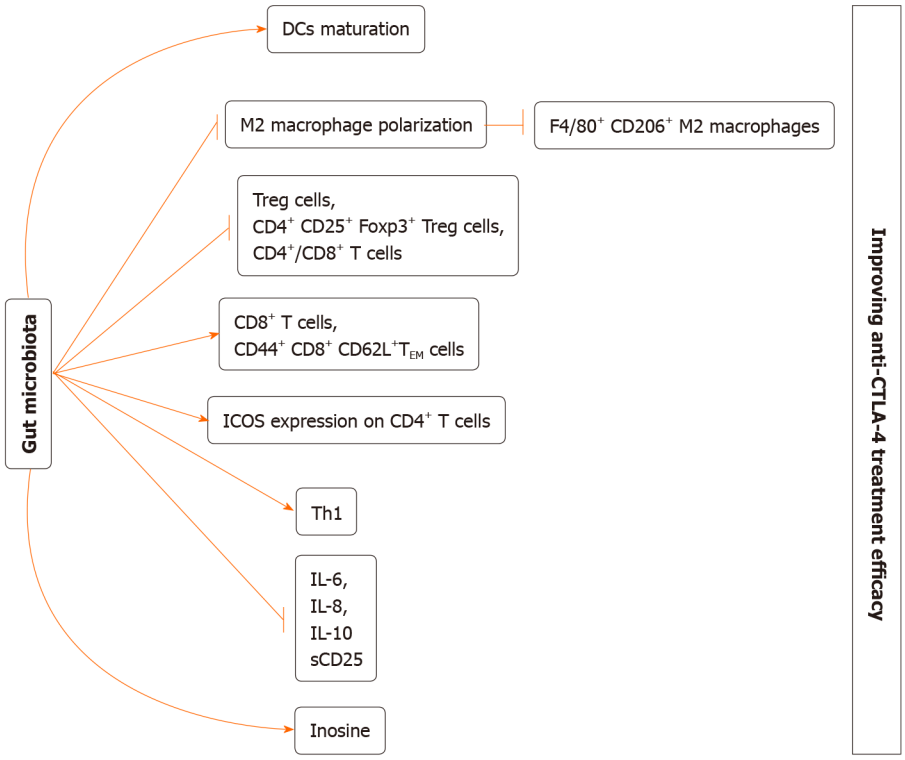


Figure 2 The potential mechanism of gut microbiome regulating anti-cytotoxic T-lymphocyte-associated protein 44 treatment efficacy. (1) Gut microbiota may induce dendritic cells maturation; (2) Gut microbiota may inhibit the M2 polarization, thereby decreasing M2 macrophages (F4/80$^+$CD206$^+$); (3) Gut microbiota may decrease regulatory T cells, CD4$^+$ CD25$^+$ Foxp3$^+$ regulatory T cells, and CD4$^+$/CD8$^+$ T cells; (4) Gut microbiota increase CD8$^+$ T cells and CD44$^+$ CD8$^+$CD62L$^+$ effector memory T cells; (5) Gut microbiota may increase inducible T cell co-stimulator expression on CD4$^+$ T cells; (6) Gut microbiota may induce T helper 1 immune response; (7) Gut microbiota may reduce interleukin (IL)-6; IL-8; IL-10, and sCD25 level; and (8) Gut microbiota may increase inosine production. Altogether, all of these approaches may eventually improve anti- cytotoxic T-lymphocyte-associated protein 4 treatment efficacy. DCs: Dendritic cells; Treg: Regulatory T; T_{EM}: Effector memory T cells; ICOS: Inducible T cell co-stimulator; sCD25: Soluble CD25; IL: Interleukin.

nivolumab were related to the shift of microbial composition. For establishing the cause-effect relation of gut microbial composition with the anti-PD-1 therapy efficacy, some preclinical studies discovered that RCC-bearing mice receiving FMT from RCC cases developed resistance to anti–PD-1 therapy (NR-FMT). At the same time, both beneficial commensals (*Bacteroides salyersiae* and *A. muciniphila*) verified through whole genome sequencing and FMT successfully compensated the NR-FMT mice.

Conforming to the above results, Gopalakrishnan *et al*[67] evaluated the gut microbiota in melanoma cases who received the PD-1 blockage treatment (faecal samples from 43 cases, including 13 non-responders and 30 responders). As a result, responders exhibited a greater gut microbial diversity. Besides, in faecal samples, α-diversity showed positive correlation with PFS[67]. Further analysis indicated that the level of Clostridiales/Ruminococcaceae, *Faecalibacterium* (belonging to the *Ruminococcaceae* family, *Clostridiales* order) was higher in responders, while *Anaerotruncus colihominis*, *Bacteroides thetaiotaomicron* (belonging to *Bacteroidales* order), and *Escherichia coli* were significantly enriched in non-responders[67]. Additionally, the abundance of *Faecalibacterium* and Bacteroidales showed positive and negative relationships with tumour infiltrating CD8$^+$ T cell level, respectively. The high abundances of *Faecalibacterium*, *Ruminococcaceae*, and *Clostridiales* in peripheral blood were accompanied with the increased effector T cell proportion, whereas *Bacteroidales* abundance showed positive relationship with regulatory T cells (Tregs) and myeloid-derived suppressor cell proportions. As revealed by multiple immunohistochemistry analyses, there were greater abundances of immune markers enriched in *Faecalibacterium* of the melanoma cases[67]. Such conclusions were verified by FMT experiments carried out in mouse models[67]. Matson *et al*[68] also found that gut microbiota affected anti-PD-1 therapy efficacy among the melanoma cases who developed metastasis. Meanwhile, as suggested by gut microbial analysis, the abundances of *B. longum, Bifidobacterium adolescentis, Enterococcus faecium, Collinsella aerofaciens, Lactobacillus* species, *Klebsiella pneumoniae, Veillonella parvula*, and *Parabacteroides merdae* were higher among responders, whereas those of *Roseburia intestinalis* and *Ruminococcus obeum* were significantly higher among the non-responders[68]. In addition, germ-free (GF) mice

subjected to gavage with responders-derived faecal materials showed significantly elevated IFN-γ and $CD8^+$ TIL levels and suppressed tumour growth, which facilitated to form the immunosupportive microenvironment[68]. Meanwhile, Routy *et al*[69] suggested that gut microbiota played a certain role in the resistance to anti-PD-1 therapy. Investigators discovered that, cancer cases who received anti-PD-1 therapy with antibiotics treatment had markedly reduced overall survival and PFS relative to those with no antibiotic treatment.

For investigating the association of antibiotic-induced dysbiosis with the reduced efficacy, investigators compared the compositions of gut microbiota in responders with those in non-responders[69]. Across the enriched bacterial species in the responders, *A. muciniphila* showed the highest correlation with the response rate of patients[69]. In addition, the IFN-γ production-induced immune reactions between Tc1 and Enterococcus hirae as well as between Th1/Tc1 and *Akkermansia muciniphila* predicted better patient survival[69]. In addition, clinical trial conducted using the mouse model suggested that mice that received FMT from responders showed superior response to the anti-PD-1 therapy and had higher proportion of $CXCR3^+CD4^+$ TILs, whereas those that received FMT from non-responders, underwent antibiotic treatments and those in the GF status developed resistance to anti-PD-1 therapy[69]. Interestingly, antibiotic treatment reversed the efficacy of PD-1 blockade treatment through *A. muciniphila* recolonization in the presence or absence of *Enterococcus hirae*. Administration of *E. hirae* and *A. muciniphila* through oral gavage can increase $CCR9^+$ $CXCR3^+CD4^+$central memory T cells, promote IL-12 and IFN-γ secretion, and increase the CD4/Foxp3 ratio within tumour bed[69].

Role of gut commensals in the anti-CTLA-4 therapy

CTLA-4 is also a research hotspot apart from PD-1/PD-L1. The anti-CTLA-4 therapy can reverse the CTLA-4-hijacked activity of the co-stimulatory signal transduction pathway (CD28-CD80/86). Therefore, it is important to identify factors that modulate the anti-CTLA-4 therapy efficacy, so as to mitigate drug resistance and promote the treatment response.

Vétizou *et al*[70] carried out a trial for investigating the gut microbial impact on the efficacy of anti-CTLA-4 therapy[70]. In the mouse model of MCA205 sarcomas, compared with GF mice and those receiving broad-spectrum antibiotic treatment, specific pathogen-free mice showed higher efficacy in anti-CTLA-4 therapy[70], and commensal flora perturbation was observed after anti-CTLA-4 therapy. For certain species (*B. uniformis* and *Bacteroides thetaiotaomicro*n), their abundances increased, while those of *Burkholderiales* and *Bacteroidales* declined[70]. Notably, *Bacteroides fragilis*, which was verified to be the immune-modulating bacteria, remained almost unchanged in the process of treatment[70,71]. Additionally, *B. thetaiotaomicron, Burkholderia cepacian*, and *B fragilis* recolonization in GF mice or those receiving antibiotic treatment reversed the resistance to anti-CTLA-4 therapy[70]. Moreover, it was further detected that *B fragilis* administered by oral gavage promoted DC maturation and elicited Th1 immune response within the tumour-draining lymph nodes[70]. Furthermore, adoptive Th1 cell transfer of cells specific to *B. fragilis* reversed the anti-CTLA-4 sensitivity in GF mice or those receiving antibiotic treatment to some extent[70]. In addition to the promoted anti-CTLA-4 effect, the treatment-related colitis was also alleviated by recolonizing *Burkholderia cepacia* and *B. fragilis* [70]. By FMT from melanoma cases, investigators discovered that the high abundance of *B. fragilis* was associated with tumour regression[70]. In addition, it was interesting to find that vancomycin treatment enhanced the ipilimumab efficacy, while alleviating side reactions that were not parallel to the promoted efficacy. To explore the reason, vancomycin might show indirect effect on promoting the abundance of Bacteroidales through suppressing Clostridiales proliferation[70].

Nonetheless, another trial examining the association of baseline gut microbiome with the clinical outcomes among the melanoma cases who developed metastasis came to different results from those obtained by Marie Vétizou[72]. Different from the results obtained from the clinical trial on mouse models, the low baseline abundances of *B. thetaiotaomicron* and *B. fragilis* but high abundance of *Bacteroides* were detected among the enrolled cases, which restricted the anticancer activity of CTLA-4. In addition, certain Firmicutes species, such as unclassified *Ruminococcus*, *Faecalibacterium* genus, *Clostridium XIVa*, *Lachnospiraceae* genus, *Gemmiger formicilis*, butyrate producing bacterium, and *Blautia* were associated with the increased response rates and superior clinical outcomes (prolonged overall survival and PFS). For exploring the underlying mechanisms, parameters associated with the immune status were analyzed, which suggested that cases exhibiting increased response to treatment had reduced baseline proportions of systemic proinflammatory cytokines (sCD25, IL-6, IL-8), $CD4^+/CD8^+$ T

cells, and Tregs while increased inducible T cell co-stimulator level in $CD4^+$ T cells. Different from the above-mentioned clinical trials, antibiotic treatment made no difference to the composition of predominant microbiota or bacteria that potentially affected the efficacy[72]. It was previously suggested that antibiotic treatment reduced the efficacy of ICI treatment, and such findings should be further investigated. Such different results among different trials might be associated with certain factors such as the heterogeneities between human and mouse models and the bias in FMT.

Recently, Zhuo *et al*[73] assessed the protection of anti-CTLA-4 blocking Ab (CTLA-4 mAb) in combination with *Lactobacillus acidophilus* cell lysates in the syngeneic BALB/c mouse model with CRC. Compared with CTLA-4 mAb monotherapy, the body weight loss was mitigated by *L. acidophilus* lysates. Meanwhile, CRC growth was suppressed in mice receiving combined administration, suggesting the effect of lysates on enhancing the anticancer effect of CTLA-4 mAb detected using the mouse model [73]. Such improved therapeutic effect was related to the higher proportions of effector memory T cells ($CD44^+CD8^+CD62L^+$) and $CD8^+$ T cells, but the lower proportions of M2 macrophages ($F4/80^+CD206^+$) and Treg ($CD4^+CD25^+Foxp3^+$) cells within TME[73]. Additionally, *L. acidophilus* lysates showed a certain immunomodulatory activity by inhibiting IL-10 expression in lipopolysaccharide-activated Raw264.7 macrophages and M2 polarization[73]. Finally, faecal microbiota was subjected to 16S ribosomal RNA gene sequencing, demonstrating that combined administration markedly suppressed the abnormally increased proteobacteria abundance and partially offset the CRC-caused dysbiosis among the model mice[73]. Consistently, Mager *et al*[74] isolated three bacterial species—*Bifidobacterium pseudolongum*, *Lactobacillus johnsonii*, and *Olsenella* species—that significantly enhanced the efficacy of anti-CTLA-4 treatment in CRC mouse models. Based on further research, intestinal *B. pseudolongum* improved immunotherapy response by producing the metabolite inosine. Decreased gut barrier function induced by immunotherapy enlarged systemic translocation of inosine and activated antitumour T cells. The effect of inosine relied on T cell expression of the adenosine A2A receptor as well as the required co-stimulation.

In general, alterations of intestinal bacteria exert a significant influence in ensuring the efficacy of cancer with ICIs treatments, with specific changes of the commensal microbes standing for a potential way that can be used to improve or to weaken ICIs efficacy. As a result, manipulating gut microbiota composition may provide a direct and effective method to strengthen the therapeutic effect of cancer ICIs.

FUTURE PROSPECTS OF FMT COMBINED WITH ICIS THERAPY IN CANCER TREATMENT

FMT refers to the process where the faecal suspension obtained from a normal subject is injected to the gastrointestinal tract of another subject for the sake of curing a certain disease. FMT is a direct and superior approach to enhance the efficacy of ICIs through modulating the gut microbiota in human beings. FMT has been adopted for more than 50 years. Faeces was initially adopted by Ge Hong in China in the 14^{th} century to treat various conditions, such as diarrhea[75]. Eiseman *et al*[76] adopted faecal enemas to treat pseudomembranous colitis in 1958 [probably because of *Clostridium difficile* infection (CDI)], and this was also the first time to introduce FMT to the mainstream medicine. Thereafter, FMT has become more and more popular because of its simple use and effects on treating CDI. In recent years, FMT has been investigated in numerous other fields[77]. FMT has been found to be effective on certain disorders, like irritable bowel syndrome, inflammatory bowel disease, anorexia nervosa, metabolic disorders, multiple sclerosis, autoimmune disorders cancer, cardiovascular diseases, and neuropsychiatric disorders[78-82]. Similarly, FMT may represent an efficient approach to increase response rate in ICIs therapy. Currently, only a few clinical trials have studied the effects of FMT on PD-1 Ab immunotherapy response in cancer patients. Recently, Baruch *et al*[83] carried out one phase I trial for evaluating whether it was safe and feasible to perform FMT and re-induction of PD-1 blockage treatment among 10 melanoma cases who developed PD-1-refractory metastases (Table 2). Clinical responses were detected among 3 cases, including 2 with partial responses and 1 with complete response[83]. Obviously, treatment with FMT showed association with favorable changes in immune cell infiltrates and gene expression profiles in both the gut lamina propria and the TME[83]. Additionally, an ongoing single-center phase 2 clinical trial (NCT03341143) investigates the therapeutic effect of FMT combined with pembrolizumab on melanoma patients who develop resistance to the anti-PD-1 treatment[84]. However, results are not reported at present. In

conclusion, such preliminary results shed more lights on the effect of FMT on anticancer treatment. As a result, FMT combined with ICIs has been regarded as the potential anticancer treatment.

CONCLUSION

Accumulating evidence has demonstrated the shift in gut microbiome composition influencing ICIs efficacy. Nevertheless, it obviously shows that in-depth studies on the mechanism(s) of interaction between gut microbiota and ICI efficacy need to be performed in different caner populations. Additionally, FMT combined with ICIs may serve as a new anticancer treatment that requires more investigation. Scientific research in this field is just at the beginning stage, and more relevant information is needed. To this end, first of all, it is of great importance to determine the mechanism by which FMT re-establishes the balanced gut microbiota, finally achieving the remarkable cure rate among cancer cases who receive ICI therapy. Secondly, well-designed randomized controlled trials are required to ensure the safety and efficacy of FMT for cancer patients with ICIs treatment. Besides, additional high-quality data (*e.g.*, longitudinal study) are also necessary to explore potential adverse effects. Moreover, it is of importance to study the composition of the small intestinal and faecal microbiota before and after FMT. These studies can contribute to better understanding the mechanisms of this therapy as well as identify microbes and their products involved in the pathogenesis of cancer. Thirdly, the best gut microbiota composition to enhance ICIs efficiency need to be recognized. On this basis, it is important to choose the right donors. Finally, FMT represents a relatively simple procedure during short duration. Compared with the repeated hospitalization and conventional therapy, FMT has low costs. Thus, the most appropriate method and duration for FMT needs to be determined. For this reason, besides conventional approaches, FMT is promising as an alternative therapy for cancer in the future.

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MINIREVIEWS

Immune checkpoint inhibitor-related hepatotoxicity: A review

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Abstract

The application of immune checkpoint inhibitors (ICI) in advanced cancer has been a major development in the last decade. The indications for ICIs are constantly expanding into new territory across different cancers, disease stages and lines of therapy. With this increased use, adverse events including immune checkpoint inhibitor-related hepatotoxicity (ICH) have emerged as an important clinical problem. This along with the introduction of ICI as first- and second-line treatments for advanced hepatocellular carcinoma makes ICH very relevant to gastroenterologists and hepatologists. The incidence of ICH varies between 1%-20% depending on the number, type and dose of ICI received. Investigation and management generally involve excluding differential diagnoses and following a stepwise escalation of withholding or ceasing ICI, corticosteroid treatment and adding other immunosuppressive agents depending on the severity of toxicity. The majority of patients with ICH recover and some may even safely recommence ICI therapy. Guideline recommendations are largely based on evidence derived from retrospective case series which highlights a priority for future research.

Key Words: Immunotherapy; Immune checkpoint inhibitors; Hepatitis; Adverse drug event; Drug-induced liver injury; Immunosuppression

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Core Tip: Immune checkpoint inhibitor (ICI)-related hepatotoxicity (ICH) is an increasingly encountered clinical problem for gastroenterologists. Although the diagnosis should be suspected in patients receiving ICI with liver function test derangements, a thorough history, examination and targeted liver investigations including cross-sectional imaging should be performed to exclude differential diagnoses. Once ICH is confirmed, its severity should be graded which then guides management. Management of ICH follows a stepwise approach beginning with cessation of ICI, followed by corticosteroids and other immunosuppressants with close monitoring after each step. The decision to recommence ICI after recovery is made on a case-by-case basis.

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INTRODUCTION

Immunological recognition of cancer cells involves an antigen presenting cell binding and presenting tumor antigens to a T cell *via* its T-cell receptor. The ensuing immune response depends on the presence of either co-stimulatory molecules (which results in immune activation) or inhibitory molecules (which results in immune downregulation and exhaustion). These inhibitory (so called checkpoint) molecules exist as a 'brake' to prevent excessive immune activation or autoimmunity. However, they are also exploited by tumor cells to evade detection by the immune system and facilitate tumor progression[1].

Immunotherapy has recently revolutionized the treatment of advanced malignancy. It involves the use of monoclonal antibodies to inhibit these immune checkpoint molecules leading to immune activation against cancer cells. The main immune checkpoint molecules currently targeted by immunotherapy are the programmed cell death protein 1 (PD-1), programmed death-ligand 1 (PD-L1), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). Since the CTLA-4 inhibitor ipilimumab was approved for the treatment of metastatic melanoma in 2011[2], immunotherapies have been trialled in many other malignancies with varying success. With their increased use, the toxicities of immunotherapies have also emerged as an important clinical problem. As expected, the side effect of enhancing the body's immune response to cancer is unwanted inflammation. These immune related adverse events (irAEs) can affect almost any organ but most commonly the skin, liver, gastrointestinal tract and endocrine glands. Treatment of irAEs may require temporary or permanent cessation of immunotherapy with or without commencement of immunosuppression which is suboptimal (at least theoretically) in terms of anti-cancer effect.

For gastroenterologists and hepatologists, immunotherapy-related hepatotoxicity deserves special mention as it is commonly encountered. Furthermore, checkpoint inhibitors have recently been added to first- and second-line treatment options for advanced hepatocellular carcinoma (HCC) after promising clinical trial results[3-5]. These patients tend to have relatively limited liver reserve since most HCCs occur on a background of liver cirrhosis[6]. Thus, immunotherapy-related hepatotoxicity can result in liver failure and even death.

In this review, we summarize the latest literature on epidemiology, pathophysiology, diagnosis, assessment and management of immune checkpoint inhibitors (ICI)-induced hepatoxicity (ICH).

EPIDEMIOLOGY OF ICH

Incidence

Although ICH is primarily considered a 'hepatitis' presenting with elevated transam-

inases, cases of immune-mediated 'cholangitis' with elevated biliary enzymes are generally also included in its definition. Further complicating the diagnosis of ICH (and therefore measurement of its incidence) is that ICI is increasingly being used in combination with other chemotherapies and targeted therapies which can themselves can cause hepatotoxicity. Indeed, distinguishing between ICH and drug-induced liver injury (DILI) secondary to co-administered therapy can be difficult.

The reported incidence of ICH depends on the severity grade [according to common terminology criteria for adverse events (CTCAE), discussed further below], type and dose of ICI and if it was used as monotherapy or in combination with another ICI. The reported incidence of all ICH (any grade) varies widely between 0%-30% while only 0%-20% experience severe ICH (grade 3 or 4) (Table 1). Fulminant hepatic failure and death following ICI has also been reported with an incidence of up to 0.4%, particularly with CTLA-4 inhibitors[7]. Indeed, ICH is overrepresented in cases of severe irAEs and accounts for 16% of all immunotherapy-related fatalities[8].

Among patients receiving monotherapy, CTLA-4 inhibitors (ipilimumab and tremelimumab) have the highest rate of hepatoxicity[9]. The incidence of ICH following CTLA-4 inhibitor monotherapy ranges between mostly between 0%-30% (mostly 3%-15%) with 1%-20% (mostly 1%-10%) being grade 3/4 in severity[2,10-12]. The frequency of ICH appears to increase when higher doses of CTLA-4 inhibitors are administered. For ipilimumab, reported rates of hepatitis (any grade) were only 3%-5% in patients treated with standard doses (3 mg/kg) compared to 15%-16% in those treated with high dose (10 mg/kg)[13,14].

In large trials of PD-L1 inhibitors (*e.g.*, atezolizumab, durvalumab, avelumab) patients exhibited rates of ICH similar to those observed for CTLA-4 inhibitors (1%-17% overall, 3%-5% grade 3/4)[15-17]. In contrast, monotherapy with PD-1 inhibitors (*e.g.*, nivolumab, pembrolizumab) results in the lowest incidence of ICH with rates consistently reported between 0%-3% (mostly 1%-2%) and very few grade 3/4 reactions (< 1%) (Table 1). Unlike CTLA-4 inhibitors, the incidence of irAEs secondary to PD-1 and PD-L1 inhibitors does not appear to be dose-related[18].

Combination therapy with CTLA-4 and PD-1 blockade has been shown to have synergistic anti-tumor effect in advanced melanoma[19]. As expected, combination therapy also results in higher rates of irAEs including ICH. In this group, ICH of any grade occurs in 18%-22% overall with 8%-11% having severe hepatitis (Table 1).

As experience with ICI increases, real-world observational data on ICH have emerged and the incidence appears to be comparable to rates reported in clinical trials[20,21].

Other risk factors

The type of underlying malignancy appears to impact on the risk of developing ICH. A meta-analysis of 17 phase II and III trials of ICI in advanced cancer found a higher likelihood of hepatotoxicity in melanoma patients compared to other types of cancer (odds ratio 5.66 *vs* 2.71, respectively)[22].

A small study of patients with pre-existing autoimmune disease observed high rates of irAEs (33% experienced grade 3-5 events) and exacerbations of autoimmune disease (27% experienced flares requiring treatment) following ipilimumab treatment[23]. However, none of these patients had autoimmune liver conditions nor experienced ICH. In a larger study of patients with pre-existing autoimmune conditions receiving anti-PD-1 therapy, rates of ICH were similar to those reported in studies of general patients (3% overall, 2% grade 3/4)[24]. Again, none of these patients had a liver-specific autoimmune condition. Unsurprisingly, irAEs are common when a patient with previous irAEs is rechallenged. However, irAE encountered after one ICI does not seem to predict the same irAEs when switching to a different class of ICI[24].

From trials in advanced HCC, ICI appears to be generally well-tolerated in patients with underlying liver disease. Although elevated aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) were commonly reported adverse events in this population (15%-23% any grade, 6%-13% grade 3/4), the proportion attributable to ICH were similar to those seen in trials of patients without liver disease[3-5]. However, trials in patients with liver disease included primarily those with compensated (Child-Pugh class A) cirrhosis, thus limiting the generalizability of these findings to sicker patients. Reassuringly, sub-analyses of a small number of patients with Child-Pugh class B7-8 (decompensated) cirrhosis in the Checkmate-040 study (n = 49) revealed overall ICH rates of 8% following nivolumab treatment[25]. Thus, from limited initial data of patients with Child-Pugh class A or early Child-Pugh class B cirrhosis, the incidence of ICH does not appear to be significantly higher than in those without liver disease.

Table 1 Incidence of severe hepatotoxicity by immunotherapy agent used as reported in key phase II and III clinical trials

Class	Agent	Ref.	Indication	Incidence of hepatoxicity (all grades) % (no. of patients)	Incidence of ≥ grade 3 hepatoxicity % (no. of patients)
CTLA-4	Ipilimumab (standard dose) 3 mg/kg	Hodi *et al*[71], 2018	Melanoma	0.3 (1/311)	0 (0/311)
		Weber *et al*[72], 2009	Melanoma	15.5 (9/58)	10.3 (6/58)
		Hodi *et al*[2], 2010	Melanoma	3.8 (5/131)	0 (0/131)
			Melanoma	2.1 (8/380) Ipilimumab with gp100	1.1 (4/380) pilimumab with gp100
		Wolchok *et al* [73], 2010	Melanoma	26.4% (19/72)	0 (0/72)
		Robert *et al*[74], 2011	Melanoma	29.1 (72/247)	20.6 (51/247)
	Ipilimumab (high dose) 10 mg/kg	Wolchok *et al* [73], 2010	Melanoma	70.4 (50/71)	15.5 (11/71)
	Tremelimumab	Ribas *et al*[75], 2013	Melanoma	0.6 (2/325)	0.6 (2/325)
Anti-PD-1	Nivolumab	Hodi *et al*[71], 2018	Melanoma	0.3 (1/313)	0.3 (1/313)
		Weber *et al*[76], 2017	Melanoma	1.9 (11/576)	0.7 (4/576)
		Brahmer *et al* [77], 2015	Squamous cell NSCLC	1.5 (2/131)	0 (0/131)
		Borghaei *et al* [78], 2015	Non-squamous NSCLC	3.1 (9/287)	0 (0/287)
		Robert *et al*[79], 2014	Melanoma	1.1 (1/89)	1.1 (1/89)
	Pembrolizumab	Robert *et al*[79], 2014	Melanoma	0 (0/84)	0 (0/84)
		Eggermont *et al* [80], 2018	Melanoma	1.8 (9/509)	1.4 (7/509)
	Cemiplimab	Migden *et al*[81], 2018	Cutaneous Squamous-Cell Carcinoma	8.5 (5/59)	0 (0/59)
Anti-PD-L1	Atezolizumab	Jotte *et al*[82], 2020	Squamous NSCLC	17.4 (58/334)	5.4 (18/334)
	Atezolizumab + Bevacizumab (anti-VEGF antibody)	Finn *et al*[3], 2020	HCC	33.4 (110/329)	10.6 (35/329)
	Avelumab	D'Angelo *et al* [83], 2020	Metastatic Merkel cell carcinoma	1.1 (1/88)	1.1 (1/88)
	Durvalumab	Garassino *et al* [84], 2018	Advanced NSCLC	0.2 (1/444)	0.2 (1/444)
Combination Therapy	Ipilimumab + Nivolumab	Hodi *et al*[71], 2018	Melanoma	3.2 (10/313)	2.6 (8/313)
		Postow *et al*[85], 2015	Melanoma	22.3 (21/94)	10.6 (10/94)
		Larkin *et al*[86], 2015	Melanoma	17.6 (55/313)	8.3 (26/313)
		Wolchok *et al* [73], 2010	Melanoma	20.8 (11/53)	11.3 (6/53)

Grading of severity is according to common terminology criteria for adverse events. CTLA-4: Cytotoxic T-lymphocyte-associated protein 4; HCC: Hepatocellular carcinoma; no. of: Number of; NSCLC: Non-small cell lung cancer; PD-1: Programmed cell death protein 1; PD-L1: Programmed death-

ligand 1; VEGF: Vascular endothelial growth factor.

High rates of allograft rejection and graft failure have been described in solid organ transplant recipients[26]. In particular, ICI treatment may be a consideration in liver transplant recipients for recurrent HCC or other malignancies (*e.g.*, melanoma). Liver injury (due to allograft rejection, not ICH) has been reported in 29%-37% of liver transplant patients in small series (n = 11-19)[26-28]. Of concern, a high proportion (3/4, 75%) of these rejections were fatal, although patient numbers were small[26,28].

PATHOGENESIS OF ICH

The exact pathogenesis of ICH is not fully understood but likely to be multifactorial. The general theory is that ICI-induced immune activation leads to not only a tumor-specific T cell response but also loss of peripheral tolerance against the patient's own cells[29]. In the case of ICH, immune activation against hepatocytes leads to a T-cell mediated hepatitis and hepatocyte death.

Mechanistically, proposed processes involved in irAEs include: T cell reactions to shared antigens expressed on both tumors and target organs, development of auto-antibodies, of ICI antibodies directly binding to target organs and/or overactivation of immune cells leading to excessive cytokine secretion[29]. The precise mechanism(s) underlying ICH development has not been elucidated.

It also remains unclear why certain patients develop ICH but not others. It has been proposed that genetic factors play an important role, however, no obvious high-risk genetic loci have been identified to date. Although it is a focus of active research, there are currently no reliable biomarkers to predict ICH in routine clinical use.

CLINICAL FEATURES

Presentation

ICH is usually asymptomatic and detected incidentally on routine liver function tests (LFTs) performed during monitoring after ICI treatment. The median time to onset of ICH is most commonly reported as 8-12 wk[14,30,31], however it can manifest as early as 2-3 wk after initiating ICI treatment[14,32-34]. Patients with more severe disease can present clinically with fever, jaundice, right-sided abdominal pain, dark urine and easy bruising. As aforementioned, acute liver failure (encephalopathy and coagulopathy) is rare, especially as the initial presentation[35]. Clinical presentations may vary depending on the class of ICI with fever found to be a more prominent feature in ICH secondary to CTLA-4 inhibitors compared to anti-PD-1 or anti-PD-L1 antibodies in one study[36].

A hepatocellular pattern of LFTs elevation (or transaminitis) is the most common pattern seen with ALT typically being higher than AST[36]. However, a cholestatic or mixed pattern of LFTs derangement is more common with PD-1/PD-L1 inhibitors compared to CTLA-4 inhibitors[36,37]. Rarely, cases of immune-mediated cholangiopathy (discussed further below) have also been described particularly with PD-1/PD-L1 inhibitors[38]. Elevations in total bilirubin can also be seen which reflects either prolonged injury or a sign of more severe disease.

Diagnosis, differentials and work-up

Although clinicians should be vigilant in looking for ICH in patients receiving ICI, not all cases of newly deranged LFTs are due to ICH. Indeed, a diagnosis of ICH only accounts for a fraction (less than 20%-30%) of all patients who were reported to have elevated AST and/or ALT as an adverse event in clinical trials[4,5,39]. Therefore, ICH is a diagnosis of exclusion and it is still important to conduct a thorough assessment to exclude other common differentials. A list of differential diagnoses with suggested investigations (or 'liver screen') is shown in Table 2.

A few key differential diagnoses deserve mention. Liver function abnormalities are more common when ICI are used in combination with other systemic chemotherapies such as dacarbazine or vemurafenib in melanoma[32], paclitaxel and carboplatin in lung cancer[40,41], or bevacizumab in HCC[3]. Furthermore, patients should be asked about other recent medications including non-prescription treatments (over-the-

Table 2 Differential diagnosis and suggested investigations

System	Differential diagnosis	Investigations
Drug-induced liver injury	(1) Other co-administered anti-cancer drugs; (2) Alcohol related liver disease; and (3) Acetaminophen toxicity	Medication history
Tumor-related	Metastatic disease	Abdominal imaging with ultrasound, CT or MRCP
Infectious	(1) Sepsis; (2) Acute HAV infection; (3) Acute HBV or flare of chronic HBV; (4) Chronic HCV; (5) Acute HEV; (6) Acute CMV or reactivation; and (7) Acute EBV	(1) Septic screen as appropriate; (2) Anti-HAV (IgM); (3) HBsAg, Anti-HBc IgG, IgM, ± HBV DNA; (4) Anti-HCV ± HCV RNA; (5) Anti-HEV (IgM); (6) CMV IgM and IgG ± CMV DNA; and (7) EBV IgM and IgG
Biliary disease	(1) Cholecystitis; (2) Cholangitis; and (3) Pancreatitis	(1) Abdominal imaging; and (2) Serum lipase
Autoimmune	Autoimmune hepatitis[1]	ANA, Anti-SMA, Anti-LKM1, serum IgG levels
Musculoskeletal	(1) Myositis (potentially an irAEs); and (2) Rhabdomyolysis	Serum CK
Metabolic	Underlying NASH	(1) Metabolic risk factors; and (2) Abdominal imaging for hepatic steatosis
Vascular	(1) Portal-vein/hepatic vein thrombosis; and (2) Ischemic hepatitis	Abdominal imaging and clinical history

[1]Although anti-nuclear antibodies is frequently positive at low-titres in IC (< 1:80), specific auto-antibodies seen in autoimmune hepatitis are usually negative.

ANA: Anti-nuclear antibodies; Anti-HBc: Anti-Hepatitis B core antibody; anti-LKM1: Anti-liver-kidney microsomal 1 antibody; Anti-SMA: Anti-smooth muscle antibody; CK: Creatine kinase; CMV: Cytomegalovirus; CT: Computed tomography; EBV: Epstein-Barr virus; HAV: Hepatitis A virus; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HEV: Hepatitis E virus; irAEs: Immune-related adverse events; MRCP: Magnetic resonance cholangiopancreatography; NASH: Non-alcoholic steatohepatitis.

counter, complementary and herbal medications) and their alcohol intake. All the above can cause a non-immune mediated (direct or idiosyncratic) DILI. Hence, a detailed medication history should be performed. Practically, it can be difficult differentiating between ICH *vs* non-ICI mediated DILI and further investigations such as liver biopsy may not reliably separate these entities.

Patients with malignancy can also have direct tumor spread to the liver or regional lymph nodes which can lead to LFTs derangement from either biliary obstruction by bulky metastases or widespread infiltration of the liver. One study of 70 patients with liver injury after receiving pembrolizumab (mainly for melanoma) found the cause of liver injury as determined by 'expert adjudication' was due to progressive hepatic metastases or malignant biliary obstruction in 60% of cases (double the number of cases judged to be due to ICH)[39]. Cross-sectional imaging with either abdominal ultrasound or more commonly computed tomography scan can be useful to identify biliary obstruction and the extent of liver metastases. As advanced cancer patients tend to get regular scans to monitor response to therapy, there may be a recent scan for review or comparison. Otherwise, imaging findings in patients with ICH are mild and non-specific even in severe cases and may include hepatomegaly, periportal edema, or periportal lymphadenopathy[42]. Extensive bone metastases can draw attention to the liver by causing elevations in total alkaline phosphatase (without a rise in gamma-glutamyl transferase)[43], which can be clarified by checking the bone-specific isoform (ostase).

Patients receiving certain systemic chemotherapies are at increased risk of reactivation of chronic hepatitis B. Although hepatitis B reactivation is very rare (< 1%) with ICI therapy[44], it can also occur with other co-administered chemotherapies or during treatment of ICH with corticosteroids. Therefore, patients should have their hepatitis B status checked routinely prior to commencing treatment with ICIs[45] and be considered for prophylactic antiviral therapy. Although a flare of hepatitis C virus (HCV) secondary to ICI therapy has not been previously reported[4,5], patients with untreated HCV should have close monitoring of their viral load and LFTs, which may both fluctuate during treatment[46]. Indeed, anti-PD1 treatment has been shown to facilitate control of chronic HCV infection[47] with some patients achieving dramatic reductions in their viral load and normalization of their ALT[46].

Role of liver biopsy

Although a liver biopsy is useful to exclude differential diagnoses and assess the degree of liver inflammation, it is usually not necessary in the work-up and ma-

nagement of ICH. Its invasive nature and cost, means it is generally reserved for situations when the diagnosis is unclear despite non-invasive investigations or the clinical course is atypical and not responding to standard treatments. Histological findings in ICH is variable but typically demonstrate lobular and/or periportal inflammation[36]. Centrilobular necrosis and central endothelialitis may also be present. Some distinct phenotypes may exist with granulomatous hepatitis reported in patients treated with CTLA-4 inhibitors, and a lobular, non-granulomatous hepatitis in those treated with anti-PD-1/anti-PD-L1 antibodies. Inflammatory infiltrates in ICH are predominantly activated T-lymphocytes (mainly CD8+) and histiocytes with few or no plasma cells[36]. Comparatively, patients with autoimmune hepatitis flares have proportionately more CD20+ B lymphocytes and CD4+ T-lymphocytes[48]. Figure 1 presents two distinct histologic presentations of ICI-induced hepatotoxicity.

Assessing causality and grading severity

Establishing causality for ICI causing hepatotoxicity begins with excluding differential diagnoses as outlined above. Formal scoring systems such as the Roussel Uclaf Causality Assessment Method (RUCAM) provide a useful framework and are often used in the DILI research setting but less so in clinical practice[49]. One problem with RUCAM is the difficulty in achieving scores to classify patients as having 'definite' or 'highly probable' DILI without rechallenging the patient with ICI which is not always safe to do. Instead the majority of patients are labelled as 'probable' DILI[39].

Once a diagnosis of ICH is judged to be the likely cause of the patient's deranged LFTs, the severity of hepatotoxicity should be graded as it determines the treatment. Most oncology clinical trials report the severity of liver injury according to the CTCAE whereas the DILI Network (DILIN) severity index is commonly used for grading DILI severity. Both grading systems take into account the patient's symptoms, degree of liver enzyme and bilirubin derangement and/or international normalized ratio (DILIN only) (Table 3). It should be noted that neither CTCAE nor DILIN systems were devised specifically for grading ICH, but rather for raised LFTs secondary to any anticancer therapy.

MANAGEMENT OF ICH

As ICH is a relatively new entity, there have been no prospective randomized controlled trials evaluating different treatment recommendations. Thus, current international treatment guidelines are all based on low level evidence (case series and expert opinion). Table 4 outlines treatment recommendations from major oncology and hepatology societies for each CTCAE grade of ICH. Importantly, treatment should be patient-centred with their active involvement in decision making throughout.

As with any other DILI, temporary or permanent discontinuation of the culprit agent should be the first step and is recommended by all society guidelines for moderate (≥ grade 2) ICH. Therefore, LFTs should be checked at baseline and prior to each dose of ICI. Since the pathogenesis of hepatotoxicity is immune-mediated, immunosuppression is the other mainstay of treatment. Although minor differences exist between guidelines, they generally all follow a stepwise algorithm involving withholding or ceasing ICI, followed by corticosteroid treatment [oral prednisolone or intravenous (IV) methylprednisolone] and further immunosuppression [mycophenolate mofetil (MMF), calcineurin inhibitors and anti-thymocyte antibodies *etc.*] depending on the initial severity and response to subsequent treatment.

Withdrawal of ICI

A considerable proportion of patients can undergo resolution of ICH without need for immunosuppressive therapy. At least three small case series (n = 6-16) have reported ICH resolution rates of 38%-50% by simply withholding the ICI even in those with grade 3/4 disease[36,50,51]. However, most society guidelines recommend starting corticosteroids for patients with ≥ grade 2 ICH, highlighting that guideline recommendations should always be applied in the appropriate clinical context. Therefore, a period of close LFTs monitoring after withholding ICI (daily to weekly depending on severity of ICH) may be useful in helping some patients avoid unnecessary immunosuppression and their related side effects. This period of observation is particularly important in cases where ICI has been co-administered with other potentially hepatotoxic drugs. Cessation of these drugs (without immunosuppression) may lead to recovery if they were the main culprit for the liver injury. Whether ICI therapy should be temporarily or permanently ceased will be discussed later.

Table 3 Comparison of common grading systems for inhibitor-related hepatotoxicity

	Grade				
	Grade 1	**Grade 2**	**Grade 3**	**Grade 4**	**Grade 5**
CTCAE [87]	(1) Asymptomatic; (2) AST or ALT > 1-3 × more than ULN; and (3) T. Bil > 1-1.5 × more than ULN	(1) Asymptomatic; (2) AST or ALT > 3-5 × more than ULN; and (3) T. Bil > 1.5-3 × more than ULN	(1) Symptomatic liver dysfunction; (2) Fibrosis on biopsy; (3) Compensated cirrhosis; (4) Reactivation of chronic hepatitis; (5) AST or ALT > 5-20 × more than ULN; and (6) T. Bil > 3-10 × more than ULN	(1) Decompensated liver function (*e.g.*, ascites, coagulopathy, encephalopathy, coma); (2) AST or ALT > 20 × more than ULN; and (3) T. Bil > 10 × more than ULN	Death due to hepatotoxicity
DILIN [73]	(1) Elevations in serum ALT and/or ALP levels; (2) T. Bil < 2.5 ULN; (3) INR < 1.5; and (4) Present with or without symptoms (fatigue, asthenia, nausea, anorexia, RUQ pain, jaundice, pruritus, rashes, or weight loss)	(1) Elevated serum ALT and/or ALP; (2) T. Bil ≥ 2.5 ULN or INR ≥ 1.5; and (3) Symptoms may become aggravated	(1) Elevated serum ALT and/or ALP; (2) T. Bil ≥ 5 ULN ± INR ≥ 1.5; (3) Symptoms are further aggravated; (4) Indication for hospitalization; and (5) No evidence of hepatic encephalopathy	(1) Coagulation abnormality indicated by INR ≥ 1.5; (2) Signs of hepatic encephalopathy; (3) T. Bil ≥ 10 ULN or daily elevation ≥ 1.0 mg/dL in 26 wk after the DILI onset; and (4) Ascites and DILI-related dysfunction of another organ	(1) Death due to DILI; and (2) Or need to receive liver transplantation for survival

ALT: Alanine Aminotransferase; AST: Aspartate aminotransferase; CTCAE: Common terminology criteria for adverse events; DILI: Drug induced liver injury; DILIN: Drug-induced Liver Injury Network; ICH: Immune checkpoint inhibitor-induced hepatoxicity; INR: International normalized ratio; RUQ: Right upper quadrant; T. Bil: Total bilirubin; ULN: Upper limit of normal.

Corticosteroids

As discussed above, commencement of corticosteroids is not essential but something to be considered depending on the severity of ICH and initial response in LFTs after withholding ICI. Oral prednisolone at a dose of 0.5-1 mg/kg of body weight is the recommended for patients with grade 2 hepatotoxicity and IV boluses of methylprednisolone 1-2 mg/kg of body weight for patients with grade 3/4 toxicity. It is important to note these dosing regimens are not derived from comparative studies but were instead extrapolated from treatment of other irAEs or autoimmune hepatitis. Indeed, higher doses of corticosteroids (> 60 mg daily of oral prednisolone) has not been shown to confer additional benefit in time to hepatitis resolution compared to lower doses[20]. A therapeutic response and eventual resolution of ICH is achieved in the majority of patients by withholding ICI and commencing corticosteroids[36]. This should be evident after 2-3 d of treatment[52]. Once a response is seen, steroids should then be tapered gradually over at least 4 wk to minimize the risk of rebound hepatitis [53]. As for other conditions, patients receiving systemic corticosteroids should be monitored for its complications including infection, hyperglycemia, and psychosis.

Refractory disease

A minority of patients either do not respond or fail to normalize their LFTs back to baseline despite corticosteroid treatment. If there is no response with oral prednisolone, IV methylprednisolone can be considered. In the setting ICH that is refractory to both oral and IV corticosteroids, MMF has been the preferred second-line agent at a dose of 500-1000 mg twice daily (Table 4). Azathioprine is the first-line steroid-sparing agent used in autoimmune hepatitis. Although its use has been reported in ICH, it is usually not favored because its immunosuppressive effect takes several months to peak and its metabolites can cause hepatotoxicity[54-56]. The main side effects from MMF are gastrointestinal upset (abdominal pain, nausea, vomiting and diarrhea) and cytopenia.

For ICH refractory to corticosteroids and MMF, collective experience is limited to case reports. Calcineurin inhibitors (tacrolimus and cyclosporine) have been used with varying success in autoimmune hepatitis and form the backbone of liver transplant rejection treatment[57]. Their mechanism of action against T cell mediated liver inflammation makes them a logical choice in treatment-refractory ICH. Calcineurin inhibitors [20,56] along with other agents including anti-thymocyte globulin therapy[58], tocilizumab[59], plasma exchange[60] and even infliximab have all been used successfully to treat steroid-refractory ICH[61]. However, infliximab is not recommended by most guidelines since it is can cause an autoimmune phenotype DILI, albeit rarely[62]. If not done prior, a liver biopsy should be strongly considered before commencing these third-line immunosuppressive agents.

Table 4 Comparison of management recommendations for inhibitor-related hepatotoxicity by major expert societies

Grade	FDA[88]	Society of Immunotherapy of Cancer[89]	American Society of Clinical Oncology [90]	European Society for Medical Oncology[91]	Australian (eVIQ Guidelines)[53]	European Association for the Study of Liver[52]
Grade 1	(1) Monitor closely; (2) Continue ICI; and (3) Investigate for other causes of hepatitis	(1) Monitor closely; (2) Continue ICI; and (3) Investigate for other causes of hepatitis	(1) Monitor closely; (2) Continue ICI; (3) Check LFTs twice a week; and (4) Investigate for other causes of hepatitis	(1) Monitor closely; (2) Continue ICI; (3) Check LFTs weekly; and (4) Investigate for other causes of hepatitis	(1) Continue ICI; (2) Monitor LFTs more closely; and (3) Investigate for other causes of hepatitis	If irAEs are excluded (unlikely or unrelated) continue therapy with close follow-up. Start symptomatic treatment
Grade 2	(1) Withhold ICI; (2) Investigate for other causes of hepatitis; (3) Start 0.5–1.0 mg/kg/d of prednisone Po until LFTs improve to < Grade 1 then wean steroids; and (4) Consider restarting when on less than equivalent of prednisone 7.5 mg/d	(1) Withhold ICI; (2) Investigate for other causes of hepatitis; (3) Start prednisone 0.5–1 mg/kg/d Po (or equivalent) with a 4-wk taper; (4) Monitor LFTs twice a week; (5) Liver biopsy optional; and (6) Resume ICI when steroids tapered to 10 mg/d and liver enzymes are grade 1 level or better	(1) Withhold ICI; (2) Investigate for other causes of hepatitis; (3) Monitor LFTs every 3 d and if no improvement start prednisone 0.5–1 mg/kg/d Po (or equivalent); and (4) Resume ICI when LFTs grade 1 level while on less than prednisolone 10 mg/d (taper over a month)	(1) Withhold ICI; (2) Investigate for other causes of hepatitis; (3) Recheck LFTs/INR every 3 d; (4) If LFTs increase on subsequent check after stopping checkpoint inhibitor, start oral prednisolone 1 mg/kg/d; (5) Once LFTs return to grade 1, start weaning steroids; and (6) Resume ICI when liver enzymes are grade 1 level or better while on less than prednisolone 10 mg/d	(1) Withhold ICI; (2) Monitor LFTs every 3 d; (3) Investigate for other causes of hepatitis; (4) Consider corticosteroid therapy for patients who are symptomatic or worsening LFTs; and (5) Resume ICI treatment once corticosteroid treatment is complete and tapered over 4 wk	(1) Skip dose and monitor liver parameters, INR and albumin twice weekly; (2) Start symptomatic treatment; (3) If abnormal liver parameters persist longer than 2 wk, start immunosuppression and discontinue the drug; and (4) Upon improvement immunotherapy could be resumed after corticosteroid tapering
Grade 3	(1) Permanently cease ICI; (2) Start prednisone at 1–2 mg/kg/d Po (or equivalent); (3) Consider imaging and liver biopsy while assessing for alternative causes of hepatitis	(1) Permanently cease ICI; (2) Monitor complete metabolic panel every 1–2 d; (3) Start prednisone at 1–2 mg/kg/d (or equivalent); (4) If refractory to steroids, consider adding MMF. Once LFTs improve, taper over 4 wk; and (5) Consider liver biopsy	(1) Permanently discontinue medication; (2) Consider hospitalization and start IV 1–2 mg/kg/d methylprednisolone; (3) If no improvement in 3 d consider adding secondary agent; (4) Monitor LFTs daily/every other day; (5) Once LFTs improve will need to wean steroids over 4–6 wk; and (6) Do not offer infliximab	(1) Withhold ICI; (2) Investigate for other causes of hepatitis; (3) Recheck LFTs/INR daily; (4) Consider hospitalization; (5) Start prednisolone 1 mg/kg/d Po if ALT/AST < 400 U/L and normal bilirubin/albumin/INR OR start IV methylprednisolone 2 mg/kg/d if ALT/AST > 400 U/L or elevated bilirubin/INR or decreased albumin; (6) Once LFTs improve to grade 2, can switch to PO steroids and wean over 4 wk; (7) Consider rechallenge at discretion of consultant; (8) If no improvement on steroids, consider adding mycophenolate and/or tacrolimus; and (9) Do not offer infliximab	(1) Permanently discontinue treatment; (2) Investigate for other causes of hepatitis; (3) Consider liver biopsy; (4) Hospitalize patient if unwell; (5) Urgent administration of start IV 1–2 mg/kg/d methylprednisoloneFor 3 d, following high dose oral prednisolone; and (6) If no improvement on steroids, urgent referral to a gastroenterologist to prescribe other steroid-sparing agents	(1) Discontinue immunotherapy and monitor liver parameters. Consider permanent discontinuation of immunotherapy; (2) Start corticosteroids (methylprednisolone or equivalent) at a dose of 1–2 mg/kg/d depending on severity; (3) If there is no response to corticosteroids within 2–3 d, MMF should be added at 1000 mg twice daily; (4) Further immunosuppression: MMF, cyclosporine, tacrolimus, anti-thymocyte antibodies; and (5) Infliximab is not recommended
Grade 4	(1) Permanently cease ICI; (2) Start prednisone at 1–2 mg/kg/d Po (or equivalent); and (3) Consider imaging and liver biopsy while assessing for alternative causes of hepatitis	(1) Permanently cease ICI; (2) Start prednisone at 1–2 mg/kg/d Po (or equivalent); (3) Monitor complete metabolic panel every 1–2 d; (4) If refractory to steroids, consider adding MMF. LFTs improve, taper over 4 wk; and (5) Consider liver biopsy	(1) Permanently discontinue medication; (2) Consider hospitalization and start IV 2 mg/kg/d methylprednisolone; (3) Add MMF if no improvement in 72 h; (4) Consider transfer to tertiary center with hepatology consultation if no improvement; (5) Do not offer infliximab; and (6) If LFTs improve, will need steroid wean over 4–6 wk	(1) Permanently discontinue medication; (2) Consider hospitalization and start IV methylprednisolone 2 mg/kg/d; (3) Formal hepatology consultation; (4) Consider liver biopsy; (5) If no improvement on IV steroids, add MMF and/or tacrolimus; (6) Do not offer infliximab; and (7) Once LFTs improve to > grade 2, can switch to oral steroids and wean over 4 wk	(1) Permanently discontinue treatment; (2) Investigate for other causes of hepatitis; (3) Consider liver biopsy; (4) Hospitalize patient if unwell; (5) Urgent administration of start IV 1–2 mg/kg/d Methylprednisolone. For 3 d, following high dose oral prednisolone; and (6) If no improvement on steroids, urgent referral to a gastroenterologist to prescribe other steroid-sparing agents	

Grading of severity is according to ommon terminology criteria for adverse events. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; FDA: United States Food and Drug Administration; ICI: Immune checkpoint inhibitor; INR: International normalized ratio; irAEs: Immune-related adverse events; IV: Intravenous; LFTs: Liver function tests; MMF: Mycophenolate mofetil; PO: Per oral.

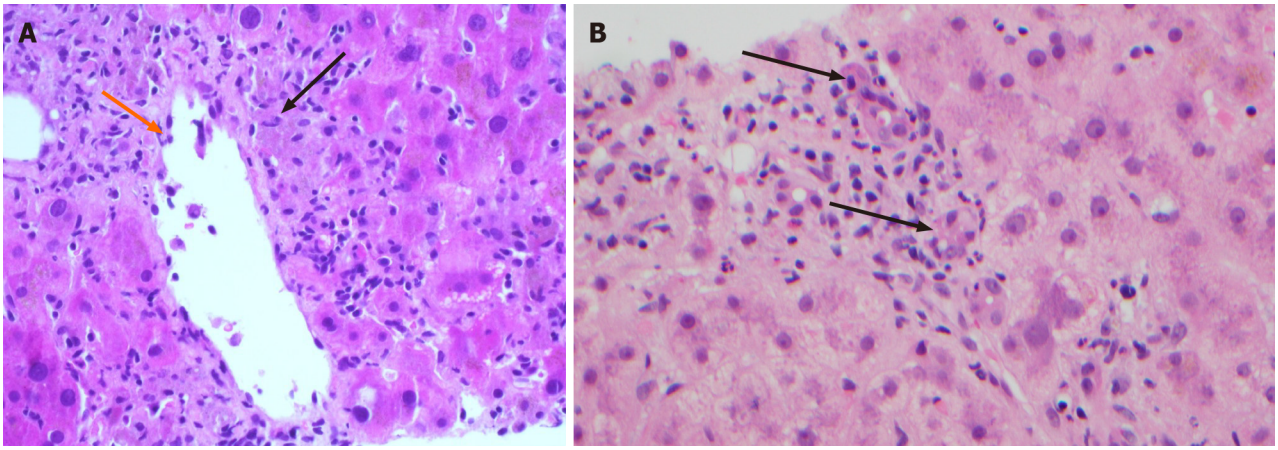


Figure 1 Histopathological changes of inhibitor-related hepatotoxicity. A: Prominent perivenulitis (black arrow) with endothelialitis (orange arrow) in a case of ipilimumab related hepatotoxicity (hematoxylin and eosin, 400 ×); B: Lymphocytic cholangitis with prominent duct damage secondary to nivolumab (black arrows; hematoxylin and eosin, 400 ×).

As aforementioned, ICH can also present as an immune-mediated cholangiopathy, especially in those receiving PD-1/PD-L1 inhibitors. In this entity, the inflammation is centred around the biliary tree with CD8+ T cell infiltration seen on liver biopsy. Characteristically has a moderate to poor response to steroid therapy[63] and the addition of ursodeoxycholic acid to immunosuppression may be helpful[64].

Ongoing monitoring and prognosis

Once a response is achieved, patients should have ongoing monitoring for clinical signs and symptoms and LFTs. The monitoring frequency (between daily to weekly) is titrated according to the severity of ICH and rate of improvement after commencing treatment[53]. Subsequent LFTs also help guide decisions regarding weaning of immunosuppression. Importantly, LFTs monitoring (fortnightly to monthly) should continue after apparent resolution of ICH and completion of immunosuppression as rebound hepatitis has been reported in up to a third of patients[65].

Although the majority of ICH patients recover and deaths due to hepatitis very rarely occur, the impact of ICH and its treatment on cancer outcomes is unclear. In a study of 491 pembrolizumab-treated patients, Tsung *et al*[39] demonstrated that development of liver injury portended worse overall survival compared to those without liver injury[39]. On sub-analysis, patients with liver injury due to 'probable' ICH exhibited significantly better survival compared to those with other causes of liver injury (*e.g.*, hepatic metastases). In contrast, corticosteroid treatment in melanoma patients with irAEs (including ICH) after CTLA-4 blockade did not impact on treatment response or survival[66,67].

As patients receiving ICI typically have advanced (incurable) malignancy, referral to palliative care services should be considered if not already done so. Palliative care services may assist clinicians and patients with managing the symptoms arising from irAEs, making treatment decisions (including when to cease therapy), and future planning. Early referral to palliative care has been shown to significantly improve patient physical and psychological symptoms, quality of life, and treatment satisfaction[68].

Restarting vs permanently ceasing ICI

The decision on whether to restart or permanently cease ICI is a difficult one. Although current guidelines recommend temporarily withholding ICI in grade 2 ICH and permanently ceasing in grade 3/4 (Table 4), the decision is often more challenging and involves weighing up the ICH (its severity and response to immunosuppression) against the advanced cancer (its current activity, response to ICI and other available treatment options). Furthermore, multiple case reports have described successful

reintroduction of ICI without ICH recurrence despite initial grade 3/4 hepatotoxicity. Other promising strategies include co-administration of budesonide (corticosteroid with high first-pass metabolism) when rechallenging with ICI[69] or resuming anti-PD-1 monotherapy in patients with severe toxicity from combination ICI therapy[70]. However, these approaches need confirmatory study before they can be widely adopted. At present, the decision to rechallenge with ICI should still be determined on a case-by-case basis through multidisciplinary discussion.

FUTURE DIRECTIONS

As the indications and frequency of ICI use expand, recognition and management of ICH will be an increasingly important issue. Current guideline recommendations are based on experience found in retrospective case series due to relatively low numbers of patients developing ICH overall (especially those who are steroid refractory). In order to strengthen the evidence base in this area, clearly both prospective large observational registries and eventually randomized controlled trials are needed. This would almost certainly involve collaborations across many centers for patient recruitment. Our rudimentary knowledge of the pathobiology behind ICH also limits our ability to predict which patients will develop it and who will respond to treatment. Therefore, efforts at the basic science level to study the genetic and immune profile of ICH patients are equally important. As our knowledge and experience increases, ICI selection may be personalized to each individual in the future not only with regards to their cancer response but also their risk of developing ICH and how best to treat it.

CONCLUSION

In summary, we have arrived at a new era of cancer therapy where ICI will form the backbone of many regimens. Uncommonly, these treatments are complicated by ICH which can be severe. After excluding key differential diagnoses, the management algorithm follows a stepwise escalation in immunosuppression. The majority of patients recover with some being able to recommence ICI therapy. Future studies are needed to boost the evidence base and hence confidence behind current treatment recommendations.

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ORIGINAL ARTICLE

Basic Study

Therapeutic effect of *Cistanche deserticola* on defecation in senile constipation rat model through stem cell factor/C-kit signaling pathway

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Abstract

BACKGROUND

Constipation is one of the chronic gastrointestinal functional diseases. It seriously affects the quality of life. *Cistanche deserticola* (*C. deserticola*) can treat constipation obviously, but its mechanism has not been clarified. We supposed that mechanism of it improved the intestinal motility by stimulating interstitial Cajal cells (ICC). Activation of the C-kit receptor on the surface of ICC is closely related to ICC function, and the stem cell factor (SCF)/C-kit signaling pathways plays an important role on it. To investigate the mechanism of how *C. deserticola* treats constipation, this study aimed to establish a constipation model in rats and explore the role of SCF/C-kit signaling pathway in the treatment.

AIM

To explore the SCF/C-kit signaling pathways in the role of *C. deserticola* for treatment of constipation by a constipation rat model.

METHODS

Forty-eight 8-mo-old Sprague–Dawley rats were divided into 4 groups by random weight method: Normal group (n = 12), model group (n = 12), *C. deserticola* group (n = 12) and blocker group (n = 12). The normal group received normal saline by gavage; the model group received loperamide by gavage; the blocker group received loperamide and *C. deserticola* by gavage, and STI571 was injected by intraperitoneally. During treatment, the weight, fecal granules and fecal quality were recorded every 10 d. On day 20 after model induction, the colon tissues of each group were removed. Hematoxylin and eosin staining was used to observe pathological changes. Expression levels of SCF, C-kit and *Aquaporin* genes were

detected by immunohistochemistry, western blotting, and real-time-quantitative polymerase chain reaction. The colonic epithelial mitochondria and goblet cells were observed by transmission electron microscopy.

RESULTS

Compared with the normal group, as treatment progressed, the weight of rats in the model and blocker groups decreased significantly, the stool weight decreased, and the stool quality was dry ($P < 0.05$). *C. deserticola* reversed the decrease in body weight and stool weight and improved stool quality. Histopathological analysis indicated that the colonic mucosal epithelium in the model group was incomplete, and the arrangement of the glands was irregular or damaged. Treatment with *C. deserticola* improved the integrity and continuity of the epithelial cells and regular arrangement of the glands. The blocking agents inhibited the effects of *C. deserticola* Immunohistochemistry and real-time-quantitative polymerase chain reaction showed that expression of SCF and C-kit protein or genes in the colonic tissue of the model group was decreased ($P < 0.05$), while treatment with *C. deserticola* increased protein or gene expression ($P < 0.05$). Western blotting showed that expression of aquaporin APQ3 was increased, while the expression of Cx43 decreased in the model group. Treatment with *C. deserticola* inhibited expression of APQ3 and promoted expression of Cx43. Transmission electron microscopy showed that the mitochondria of the colonic epithelium in the model group were swollen and arranged disorderly, and microvilli were sparse. The condition was better in the *C. deserticola* group. Mice treated with STI571 blocker confirmed that blocking the SCF/C-kit pathway inhibited the improvement of constipation by *C. deserticola*.

CONCLUSION

C. deserticola improved defecation in rats with constipation, and the SCF/C-kit signaling pathway, which is a key link of ICC function, played an important role of the treatment.

Key Words: *Cistanche deserticola*; Senile constipation; Stem cell factors; C-kit

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Core Tip: We studied a possible mechanism of *Cistanche deserticola (C. deserticola)* in the treatment of constipation. The mechanism might improve colon motility through stem cell factor (SCF)/C-kit signaling pathway in colon Cajal stromal cells. Therefore, the constipation rat model was replicated, and then rats were treated by direct administration of *C. deserticola* and specific blocking of the SCF/C-kit signaling pathway. The defecation of rats, changes of colonic pathology and ultrastructure as well as the protein expression related to the SCF/C-kit signaling pathway were observed. Our results support that the SCF/C-kit signaling pathway plays an important mechanism on the therapeutic effect of *C. deserticola*.

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INTRODUCTION

Senile constipation (SC) is one of the common chronic gastrointestinal functional diseases in geriatric patients, which induces or aggravates cerebrovascular events and other diseases and seriously affects the quality of life of the elderly population[1,2]. According to traditional Chinese medicine, the main etiology and pathogenesis of SC are gastrointestinal heat, deficiency of Yin and Jin and intestinal de-wetting[3,4], while

dataset available from the corresponding author at zhangzhen@cdutcm.edu.cn. Participants gave informed consent for data sharing.

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Cistanche deserticola (*C. deserticola*) can warm the kidneys and relax the bowels. Researchers have reported that the effect of intestinal motility improvement and treatment of SC is clear, but its mechanism has not been clarified[5,6]. Interstitial Cajal cells (ICCs) are a special type of mesenchymal cells in the gastrointestinal tract. Activation of the C-kit receptor on the surface of ICCs is closely related to cell proliferation, differentiation and function. ICCs participate in the pathogenesis of many gastrointestinal motor dysfunctions[7,8]. Activation of C-kit depends on the binding of its ligand stem cell factor (SCF)[9]. To investigate the effect and mechanism of action of *C. deserticola* on SC, this study aimed to establish an SC model in rats induced by loperidol to explore the role of the SCF/C-kit signaling pathway in the treatment of constipation by *C. deserticola*.

MATERIALS AND METHODS

Experimental materials

Experimental animals: The experimental animals were 48 male 8-mo-old Sprague-Dawley rats weighing 500-550 g; all purchased from Changsha Tianqin Biotechnology Co. Ltd. Rats were raised in the animal laboratory of Chongqing Weisiteng Biomedical Technology Co. Ltd., given normal day and night light, food and drink. All procedures complied with the management guidelines issued by the Ethics Committee of Chongqing Traditional Chinese Medicine Hospital.

Primary reagents: The primary reagents included loperamide (Sigma, St. Louis, MO, United States), *C. deserticola* from Hubei Jurui Traditional Chinese Medicine Decoction Co. Ltd., STI571 from Chinese Selleck Co. Ltd., and SCF, C-kit, connexin 43 (Cx43), aquaporin 3 (AQP3) and β-actin antibodies (Abcam, Cambridge, MA, United States), PrimeScript II 1st Strand cDNA Synthesis Kit and Premix Taq™ (Ex Taq™ Version 2.0) (Takara Co. Ltd., Japan).

Methods

SC model preparation and grouping: To avoid the influence of confounding factors such as body weight on the experimental results, the rats were randomly grouped by the block random grouping method. Forty-eight rats were fed adaptively for 1 wk, and the rats were numbered in order of weight from light to heavy. According to the order of body weight, the rats were divided into 12 zones (the weight of rats in each zone was similar), with 4 rats in each zone. The 12 zones were divided into 4 groups of 12 (normal, model, *C. deserticola* and blocker). Rats in the model group were given loperamide (0.0625/100 g, 10 mL/kg) by gavage. The first gavage dose was doubled, and the drug was given at 10:00 every day, once a day for 20 consecutive days. The normal group was given the same amount of normal saline by gavage. In addition, the model group was treated with *C. deserticola* (0.156 g/100 g, 10 ml/kg) by gavage. The blocker group was given *C. deserticola* (0.156 g/100 g, 10 mL/kg) by gavage and STI571 (25 mg/mL, 1 mL/kg) by intraperitoneal injection. During the experiment, body weight, stool weight and stool condition were recorded every 10 d. No accidental death occurred in any of the rats. Twenty days later, the rats were killed, and the colonic tissue was removed for examination and liquid nitrogen preservation. The remaining parts were fixed and embedded.

Histopathological analysis: The rats fasted overnight were anesthetized by intraperitoneal injection with 7% chloral hydrate (5 mL/kg) on the next day. The abdomen was opened to separate the colonic tissue, and 8-10 mm of colon was removed. The intestinal contents were removed by gently shaking in precooled polybutylene succinate and fixed for 24 h in 4% paraformaldehyde fixative solution. Conventional paraffin embedding was performed. Tissue sections (5 μm) were removed for hematoxylin and eosin staining, and the histopathological morphology was observed under a microscope.

Detection of expression of SCF and C-kit by immunohistochemical staining: The paraffin tissues examined by histopathology above were sectioned, rehydrated with sectioned xylene dewaxed gradient ethanol, antigen remedied by heat and sealed for 1 h with goat serum at room temperature. SCF and C-kit primary antibodies were added dropwise, and tissues were kept overnight at 4 °C. After rinsing with polybutylene succinate, horseradish peroxidase was dropped to label secondary antibody, rinsed with polybutylene succinate for 5 min, drop 2,4-diaminobutyric acid chromogen solution, re-dye with hematoxylin, reverse gradient alcohol dehydration, transpar-

entized by xylene and neutral resin seal. Microscopically, the nuclei were purplish blue, and the positive result products were brownish yellow or yellow particles.

Detection of expression of *C-kit* mRNA in colonic tissue by real-time-quantitative polymerase chain reaction (RT-qPCR): Frozen rat colonic tissue was ground by homogenizer, and 1 mL RNAiso Plus was added. Total RNA of colonic tissue was extracted by chloroform/isopropanol method, and the RNA quality was detected by gel electrophoresis. After identified by Bio-Rad gel imager, 1 μg RNA was reverse transcribed into cDNA by Takara PrimeScript II 1st Strand cDNA Synthesis Kit. According to NCBI database Gene ID: 64030, the CDS region of the *C-kit* gene was found and combined with NCBI Primer-Blast design Primer sequence, which was synthesized by Sangon Biotech Co. Ltd, *C-kit* primer sequence sense strand 5'-AGGTGTACCGTTCCTGTCCC-3', antisense strand 5'-GGCTGGATTTGCTCTTTGCTGT-3', *β-actin* sense strand 5'-CTTCCTTCCTGGGTATGGAATC-3', antisense strand 5'-CTGTGTTGGCATAGAGGTCTT-3'. PCR amplification was performed on a Bio-Rad CFX90 Real-Time PCR instrument. The conditions for PCR assay were initial denaturation at 95 °C for 5 min, 30 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min followed by final extension at 72 °C for 10 min. $2^{-\Delta\Delta Ct}$ (ΔCt = Ct value - β-actin Ct value) was taken to analyze relative gene expression.

Detection of protein expression level of Cx43 and AQP3 in colonic tissue by Western blotting: About 1 cm of frozen colonic tissue was collected and lysed with RIPA strong pyrolysis liquid (100 μL). The tissue was homogenized with a glass homogenizer 20 times and transferred to a 1.5 mL centrifuge tube. The supernatant was extracted after centrifugation at 12000 rpm at 4 °C for 5 min, and the concentration of total protein in the supernatant was determined by BCA method. We removed 25 μg denatured protein to perform sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred the proteins in the gel to polyvinylidene fluoride membrane at a constant current of 200 mA then sealed by 5% skim milk confining liquid for 2 h at room temperature. Corresponding primary antibodies were diluted with sealing fluid, incubated at 4 °C overnight, then washed the membrane resistance and added second antibodies. After adding ECL fluid, they were put under the exposure meter for test by optical density analysist.

Transmission electron microscopy: We took about 1 cm of proximal colonic tissue, fixed it in 2.5% glutaraldehyde for 24 h, dehydrated it with graded acetone, embedded it in Epon812 and cut 50 nm ultrathin sections on an ultramicrotome. The sections were stained with uranium dioxide acetate and lead citrate dyeing liquid at room temperature for 10 min. We observed colonic villi, epithelial cell mitochondria and goblet cells under a Philips Tecnai-10 transmission electron microscope.

Statistical analysis

SPSS 19.0 software was used for data analysis, and GraphPad Prism8.4 software was used for plotting the results. The Shapiro-Wilk's test was used for normality testing. Data with normal distribution were represented by mean ± SD. Error items were used to represent the mean ± SD of hemoglobin after treatment. Student's *t*-test was used for comparison between two groups. Analysis of variance was used for comparison between multiple groups. The Student-Newman-Keuls method was used for comparison between multiple groups in pairs, and Dunnett's test was used to measure the differences between each experimental group and control group in sequence. Associated data were measured by multiple time point, and repeated measurement data analysis of variance was conducted. If the sphericity test showed $P > 0.05$, repeated measurement data analysis of variance was used. If $P < 0.05$, the generalized estimation equation was taken. Data that did not conform to a normal distribution were represented by M (P25, P75), and the Kruskal-Wallis *H* rank sum test was used for comparison between groups. The χ^2 test was used to compare between groups when the data was unordered data, and Pearson's χ^2 test was used if the proportion of cells with theoretical frequency < 5 was < 20%. If the proportion of cells with theoretical frequency < 5 was ≥ 20%, Fisher's exact test was used. $P < 0.05$ was used to determine that the difference was statistically significant.

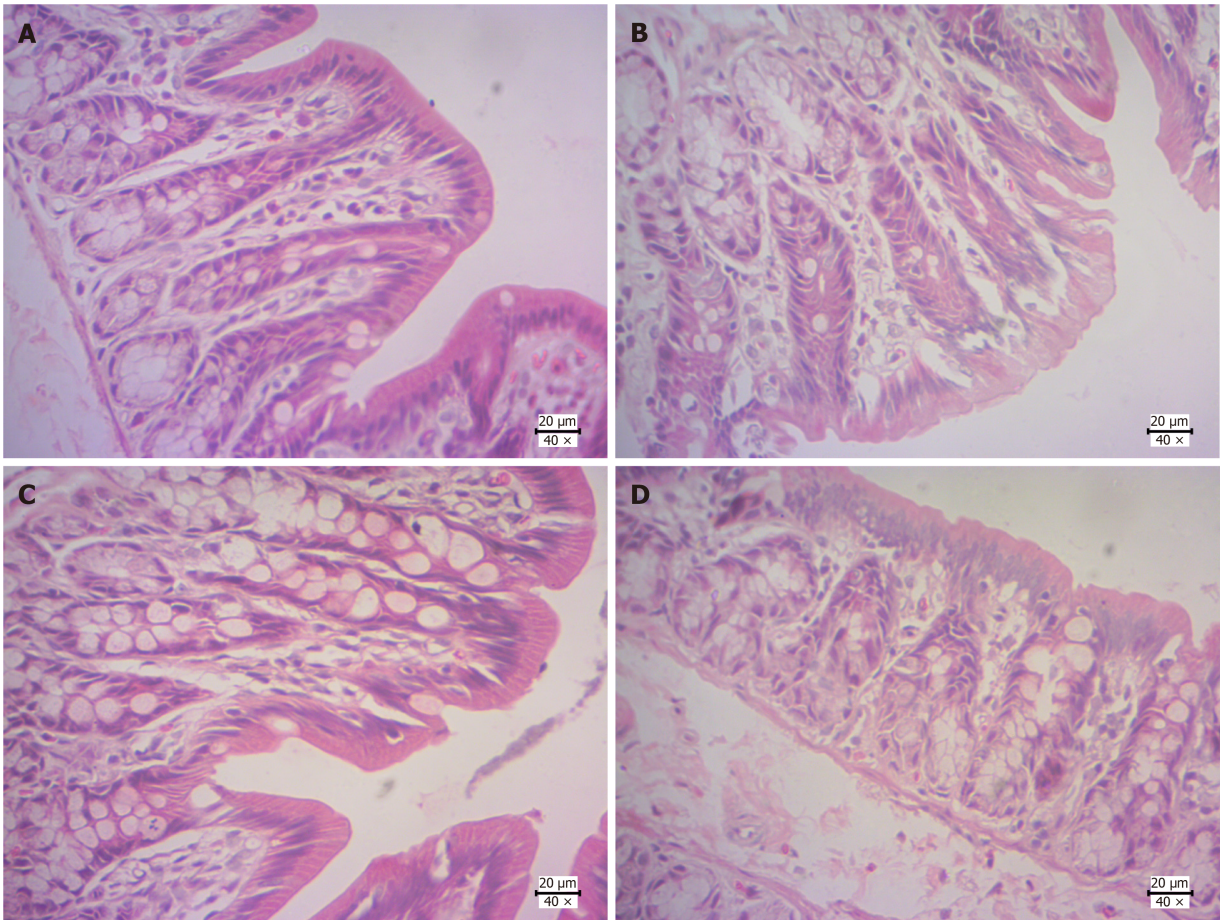


Figure 1 Colonic histopathological changes. A: Normal group; B: Model group; C: *Cistanche deserticola* group; D: Blocker group. Magnification: × 40; Scale bar: 20 μm.

RESULTS

General conditions

One day after gavage of loperamide compared with the normal group, the body weight and stool weight in the model group were significantly decreased ($P < 0.05$), but there was no significant difference in stool quality score ($P > 0.05$). *C. deserticola* treatment in the model group slowed the decrease in body weight and stool weight ($P < 0.05$), but the body weight in the blocker group was not significantly reduced. After 10 d of treatment, compared with the normal group, the body weight and stool volume of rats in the model group were further significantly reduced, and the stools were dry and almost contained no water ($P < 0.05$). The body weight loss of rats was also significantly reduced by treatment with *C. deserticola* ($P < 0.05$). The body weight of rats in the blocker group was not significantly different from that in the model group ($P > 0.05$). On day 20 before sampling, the body weight of rats in the model group was significantly lower than that in the normal group, and the stool quality score was significantly increased ($P < 0.05$), while the weight loss caused by constipation was inhibited by *C. deserticola* treatment, and the stool quality score was decreased ($P < 0.05$).

Colonic histopathology

Hematoxylin and eosin staining of colonic tissue of rats is shown in Figure 1. In the normal group, intestinal villi were intact, intestinal mucosa was intact and continuous, cells and glands were arranged regularly. Mucosa and submucosa were not infiltrated by inflammatory cells, and tissue structure was normal. In the model group, intestinal villi and mucosa were largely destroyed, local villi were shed and loosely arranged, and part of the glandular structure disappeared. In the *C. deserticola* group, histopathological staining was less than that of the model group, but there was necrosis in some goblet cells. After intraperitoneal injection in the blocker group, compared with the *C. deserticola* group, there was still local loss of intestinal villi, and there was fracture and

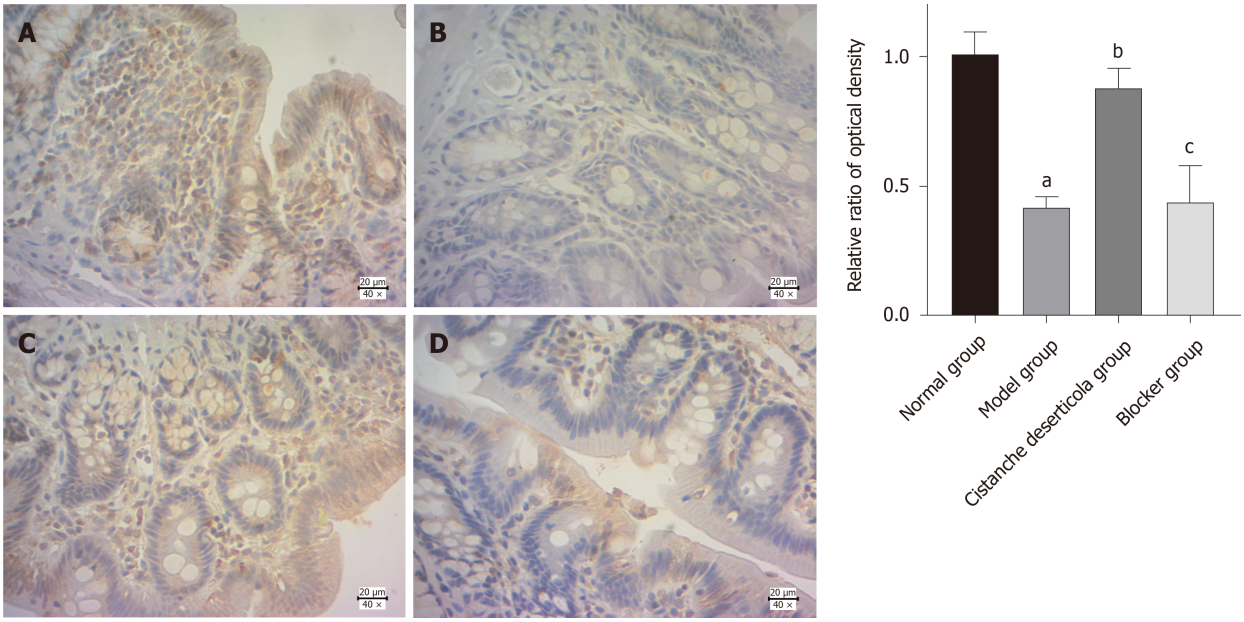


Figure 2 Expression of stem cell factor and relative optical density ratio in colonic tissue by immunohistochemical detection. A: Normal group; B: Model group; C: *Cistanche deserticola* group; D: Blocker group. Magnification: × 40; Scale bar: 20 μm. [a]*P* < 0.05, the expressions of stem cell factor and C-kit in colon tissue of the model group were both significantly decreased compared with the normal group; [b]*P* < 0.05, the expression of stem cell factor and C-kit in the colon tissue of rats in the *Cistanche deserticola* group was significantly increased compared with model group. There was no difference in the expression of stem cell factor and C-kit in the colon tissue of rats between the blocker group and the model group.

loss of intestinal mucosa.

Detection of expression of stem cell factor and C-kit protein by immunohistochemical staining

Immunohistochemical staining showed that SCF and C-kit were expressed in the colon of the control group. Compared with the control group, expression of SCF and C-kit in the model group was significantly decreased ($P < 0.05$). Compared with the model group, expression of SCF and C-kit in the *C. deserticola* group was significantly increased ($P < 0.05$). There was no difference in expression of SCF and C-kit between the blocker and model groups ($P < 0.05$, Figures 2 and 3).

RT-qPCR

RT-qPCR showed that expression of *C-kit* mRNA in the model group was significantly lower than in the normal group (Figure 4, $P < 0.05$). Compared with the model group, *C-kit* in the *C. deserticola* group was significantly increased ($P < 0.05$). There was no significant difference in *C-kit* mRNA level between the model and blocker groups ($P > 0.05$). These results indicated that *C-kit* expression adjusted by *C. deserticola* participated in the improvement of constipation, and blockers inhibited the efficacy of *C. deserticola*.

Western blotting

Western blotting showed that AQP3 protein was expressed at a low level in the normal control group, while it was significantly increased in the model group compared with the control group (Figure 5) ($P < 0.05$). Expression of AQP3 protein was decreased after treatment with *C. deserticola*, and the inhibitory effect of *C. deserticola* on the expression of AQP3 was inhibited by blocker treatment ($P < 0.05$). Expression of Cx43 was the opposite of that of AQP3. These results suggested that the pharmacology effect of *C. deserticola* on rats with SC was achieved by promoting expression of Cx43 and inhibiting expression of AQP3 (Figures 5-7).

Transmission electron microscopy

After loperamide induction, mitochondrial swelling and autophagy occurred in some of the colonic mucosal epithelial cells in the model group. The number and length of intestinal microvilli decreased, and goblet cell pyknosis occurred (Figure 8). After treatment with *C. deserticola*, the number of intestinal microvilli increased. The microvilli were arranged neatly, and the structure was complete. The goblet cells

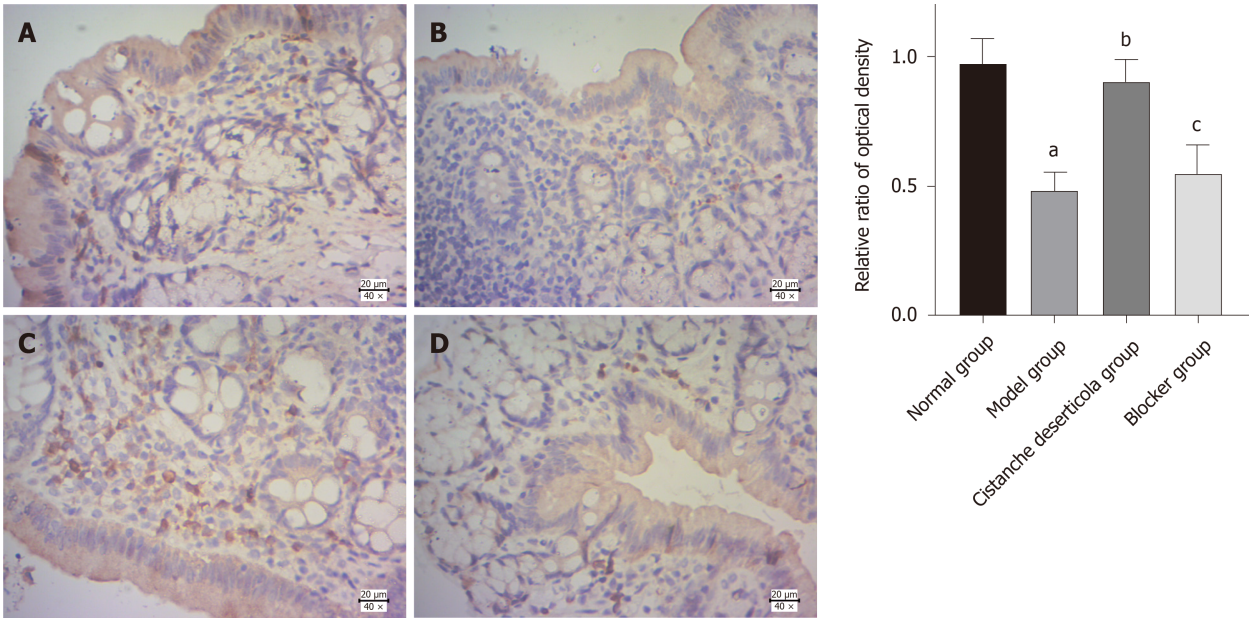


Figure 3 Expression of C-kit and relative optical density ratio in colonic tissue by immunohistochemical detection. A: Normal group; B: Model group; C: *Cistanche deserticola* group; D: Blocker group. Magnification: × 40; Scale bar: 20 μm. [a]*P* < 0.05, compared with normal group; [b]*P* < 0.05, compared with model group; [c]*P* > 0.05, compared with model group.

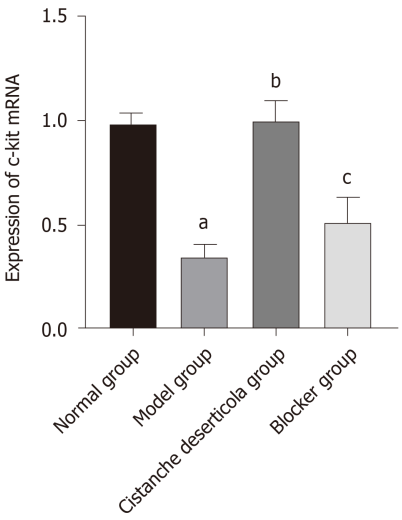


Figure 4 Expression of C-kit mRNA in colon tissue of rats in each group. [a]*P* < 0.05, compared with normal group; [b]*P* < 0.05, compared with model group; [c]*P* > 0.05, compared with model group.

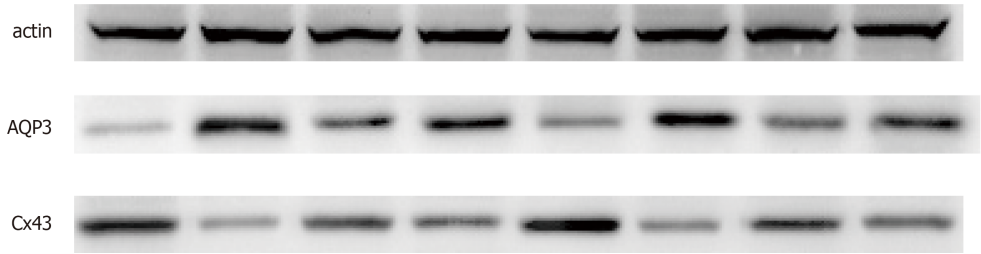


Figure 5 Connexin 43 and aquaporin 3 protein expression detected by western blotting. AQP3: Aquaporin 3; Cx43: Connexin 43.

regained some structural integrity but still did not return to normal length. After inhibitor treatment, the number of microvilli in colonic epithelial cells decreased significantly compared with those in the *C. deserticola* group, with loose arrangement,

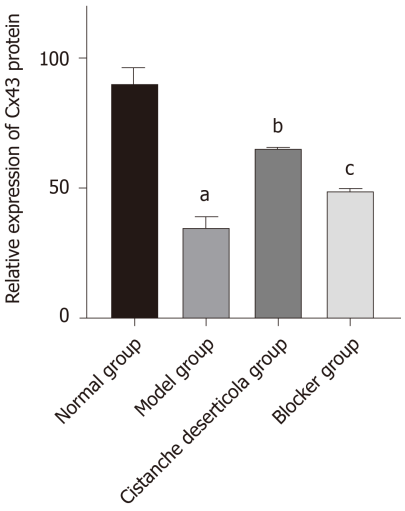


Figure 6 Relative expression of connexin 43 protein. $^{a}P < 0.05$, compared with normal group; $^{b}P < 0.05$, compared with model group; $^{c}P > 0.05$, compared with model group. Cx43: Connexin 43.

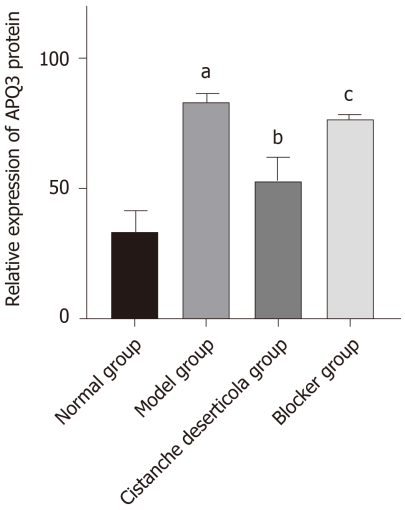


Figure 7 Relative expression of aquaporin 3 protein. $^{a}P < 0.05$, compared with normal group; $^{b}P < 0.05$, compared with model group; $^{c}P > 0.05$, compared with model group. AQP3: Aquaporin 3.

shorter length, irregular morphology of mitochondria and severe pyknosis of goblet cells.

DISCUSSION

Constipation is common in the elderly population and is one of the common symptoms that affect the health status and quality of life of elderly people[10,11]. In terms of traditional Chinese medicine, Bai *et al*[12] believed that the basic pathogenesis of SC was dysfunction of large intestine conduction function, senile body failure, gradual loss of liver and kidney functions, lack of qi and blood and loss of body fluid, which led to dry stools, resulting in constipation[13]. SC puts pressure on patients and their families in many aspects, and it is important to explore its pathogenesis.

C. deserticola has the functions of tonifying kidney Yang, benefiting blood and regulating body immunity[14]. *C. deserticola* and Chinese herbal decoction containing *C. deserticola* have been widely used in the treatment of constipation, and the clinical effect is remarkable. Du *et al*[15] found in a rat model of Yang deficiency and constipation that *C. deserticola* improved the contraction amplitude of isolated colon, strengthened the contractility of the intestinal tract and returned the level of gastrointestinal hormones to normal. In addition, dietary fiber of *C. deserticola*, a

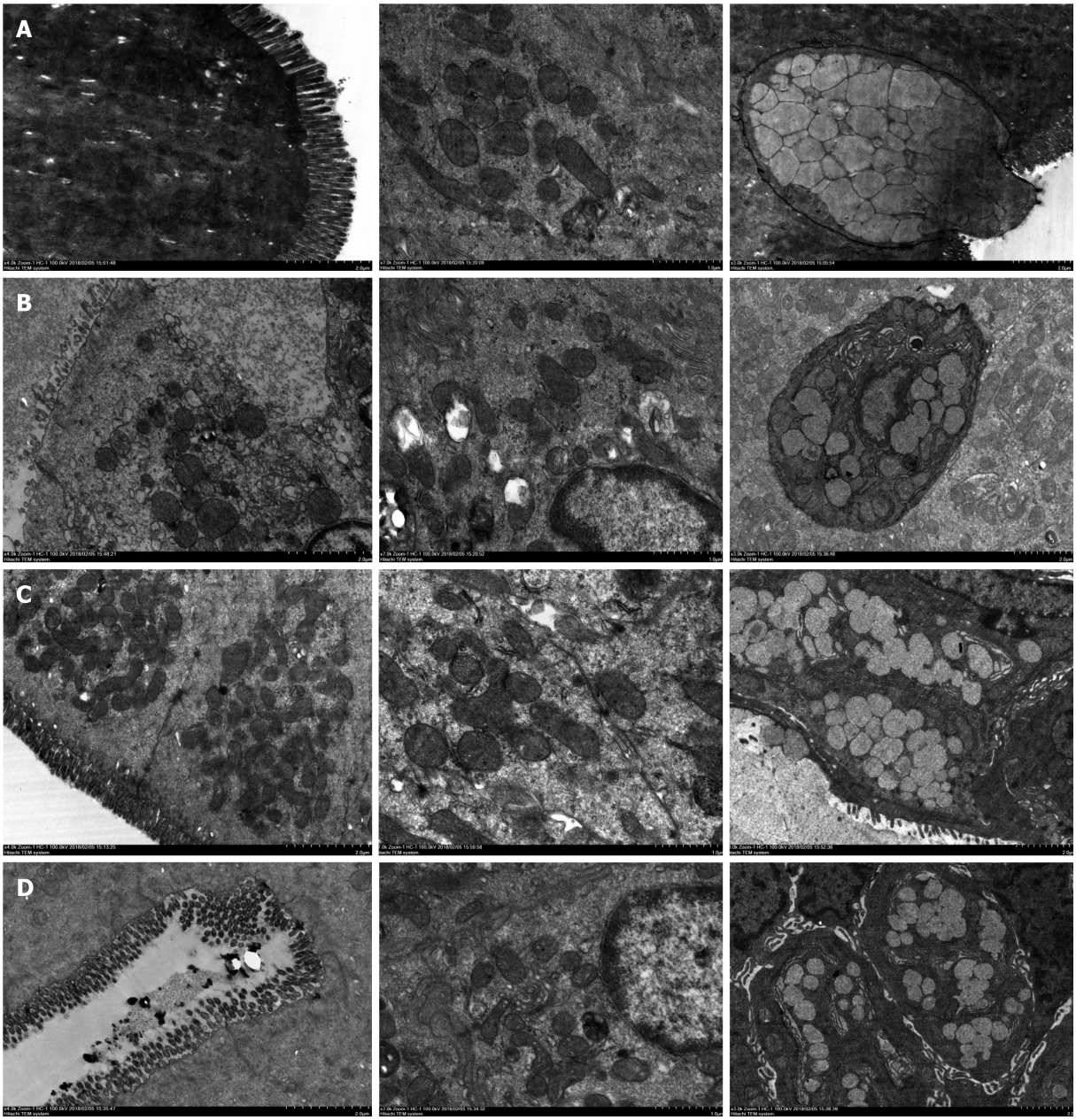


Figure 8 Observation of colonic villi, mitochondria of colonic epithelial cells and goblet cells by transmission electron microscopy. A: Normal group; B: Model group; C: *Cistanche deserticola* group; D: Blocker group.

processing byproduct of *C. deserticola*, improves the water-holding capacity of feces and has the advantage of low dose and good effect in nourishing intestines and improving defecation[16]. Our study found that *C. deserticola* treatment improved fecal moisture and colonic histopathology of rats with constipation.

ICCs are found throughout the intestinal tract and play a vital role in the neural control of intestinal movement as pacemakers and intestinal nerve transmission[17]. Proto-oncogene coding receptor tyrosine kinase, C-kit, is expressed by ICCs, and SCF is a ligand of C-kit[18]. The SCF/C-kit system is crucial in the development of ICCs. The SCF/C-kit signaling pathway has been analyzed during treatment of slow transfer constipation in rats by acupuncture[19]. Electroacupuncture at Tianshu point increased the number of colon ICCs in ACK2-treated mice, upregulated expression of SCF and C-kit proteins and reversed the phenotype of ICC. Prescription for invigorating qi and spleen can increase expression of ICC in colonic tissue and upregulate expression of *C-kit* and *SCF* mRNA, thus improving constipation symptoms[20]. In our study, immunohistochemistry showed that *C. deserticola* significantly upregulated expression of SCF and C-kit protein in a rat model of SC, and RT-qPCR showed that *C. deserticola* significantly upregulated expression of *C-kit* mRNA. C-kit blocker inhibited the upregulation of SCF and C-kit expression induced

by *C. deserticola*.

Fecal water content and colonic water transport are closely related to AQPs, which play an important role in intestinal water metabolism[21]. In a study of rats with slow transport constipation, Defecation decoction significantly improved constipation symptoms by regulating intestinal water absorption through downregulating AQP3 and AQP4[22]. In a rat model of functional constipation, the treatment mechanism of Oral Xiaofu Tongjie Fluid for functional constipation may be realized by regulating expression of vasoactive intestinal polypeptide and AQP3 in the colon[23]. Gap junctions play an essential role in mediating synchronous contraction of smooth muscle cells. Cx43 is the most important protein during that process[24]. Loss of Cx43 expression may be partially responsible for smooth muscle motor dysfunction[25]. Repair of the intestinal nervous system network structure in rats with slow transit constipation can improve the expression of Cx43 *etc.* and constipation symptoms[26]. These results indicate that the increased expression of AQP3 decreases fecal water content, leading to constipation, which may be an important mechanism of constipation. Expression of Cx43 may be related to contraction of intestinal smooth muscle and reduce the occurrence of constipation. In our study, western blotting showed that Cx43 expression was increased and AQP3 expression was inhibited by *C. deserticola* treatment, which resulted in increased smooth muscle contraction and decreased water absorption, and blocker treatment reversed these effects.

CONCLUSION

In conclusion, *C. deserticola* can inhibit expression of AQP3 and promote expression of Cx43 through the SCF/C-kit pathway, thereby improving the constipation induced by loperamine and reducing colonic tissue damage in aged rats. This study provided a good theoretical basis for clinical use of *C. deserticola*.

ARTICLE HIGHLIGHTS

Research background

Chronic constipation is a common functional gastrointestinal disease that seriously affects the quality of life, especially for senile patients. *Cistanche deserticola* (*C. deserticola*) is one kind of herb that can improve constipation obviously, but the mechanism of it is unclear. Since it increases the frequency of defecation, we suppose that its therapeutic effect is due to increased intestinal motility by an important signaling pathway, stem cell factor (SCF)/C-kit, located on the surface of interstitial Cajal cells.

Research motivation

The treatment of chronic constipation is not encouraging, and the available drugs cannot meet the clinical needs. New drugs are needed to safely increase intestinal motility and improve symptoms. New drugs should be based on in-depth studies of the mechanisms of some foods that are currently widely and safely used. *C. deserticola* is one kind of herb that has been used for thousands of years in Traditional Chinese Medicine for constipation. Benefits would be evident from more studies about the treatment mechanisms.

Research objectives

To investigate the mechanism of how *C. deserticola* treats constipation, this study aimed to establish a constipation model in rats and explore the role of the SCF/C-kit signaling pathway in the treatment.

Research methods

In the case of blank control group, a constipation rat model was first established. While these rats were treated with *C. deserticola*, a group of rats were specifically blocked from the target signaling pathway. The symptoms and defecation of these different groups of rats were observed, and the tissue and gene expression in which the target signaling pathway was located were observed to explain whether the SCF/C-kit signaling pathway plays a key role in the therapeutic effect of *C. deserticola*.

Research results

The model was successfully established, and the therapeutic effect of *C. deserticola* was also obvious. In the group where the target signaling pathway was blocked, the therapeutic effect of *C. deserticola* was significantly reduced, as reflected by histological and immunohistochemical changes as well as signal-pathway-related genes and proteins such as connexin 43, aquaporin 3 expression changes. *C. deserticola* can inhibit expression of aquaporin 3 and promote expression of connexin 43 through the SCF/C-kit pathway, thereby improving the constipation induced by loperamine and reducing colonic tissue damage in aged rats.

Research conclusions

This study provided a good theoretical basis for clinical use of *C. deserticola*. Furthermore, the SCF/C-kit signal pathway plays an important role of constipation treatment of *C. deserticola*. There is likely more mechanisms related to it, for the effect of *C. deserticola* was not blocked completely.

Research perspectives

The improvement of intestinal motility is the core point in the treatment of constipation. However, further research on intestinal dynamics is still needed. The research on intestinal dynamics of interstitial Cajal cells is still a focus of attention, but whether there is an unknown mechanism of its function is one of the directions of future research. In the meantime, herbs should also be more widely valued.

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ORIGINAL ARTICLE

Basic Study

Recombinant angiopoietin-like protein 4 attenuates intestinal barrier structure and function injury after ischemia/reperfusion

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Abstract

BACKGROUND

Intestinal barrier breakdown, a frequent complication of intestinal ischemia-reperfusion (I/R) including dysfunction and the structure changes of the intestine, is characterized by a loss of tight junction and enhanced permeability of the intestinal barrier and increased mortality. To develop effective and novel therapeutics is important for the improvement of outcome of patients with intestinal barrier deterioration. Recombinant human angiopoietin-like protein 4 (rhANGPTL4) is reported to protect the blood-brain barrier when administered

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exogenously, and endogenous ANGPTL4 deficiency deteriorates radiation-induced intestinal injury.

AIM

To identify whether rhANGPTL4 may protect intestinal barrier breakdown induced by I/R.

METHODS

Intestinal I/R injury was elicited through clamping the superior mesenteric artery for 60 min followed by 240 min reperfusion. Intestinal epithelial (Caco-2) cells and human umbilical vein endothelial cells were challenged by hypoxia/reoxygenation to mimic I/R *in vitro*.

RESULTS

Indicators including fluorescein isothiocyanate-conjugated dextran (4 kilodaltons; FD-4) clearance, ratio of phosphorylated myosin light chain/total myosin light chain, myosin light chain kinase and loss of zonula occludens-1, claudin-2 and VE-cadherin were significantly increased after intestinal I/R or cell hypoxia/reoxygenation. rhANGPTL4 treatment significantly reversed these indicators, which were associated with inhibiting the inflammatory and oxidative cascade, excessive activation of cellular autophagy and apoptosis and improvement of survival rate. Similar results were observed *in vitro* when cells were challenged by hypoxia/reoxygenation, whereas rhANGPTL4 reversed the indicators close to normal level in Caco-2 cells and human umbilical vein endothelial cells significantly.

CONCLUSION

rhANGPTL4 can function as a protective agent against intestinal injury induced by intestinal I/R and improve survival *via* maintenance of intestinal barrier structure and functions.

Key Words: Angiopoietin-like protein 4; Intestinal ischemia/reperfusion; COVID-19; Myosin light chain kinase; Intestinal barrier breakdown

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Core Tip: Improving therapy on intestinal barrier dysfunction induced by ischemia/reperfusion is still challenging. Most recently, studies indicated that patients who suffered from coronavirus disease 2019 are associated with intestinal hypoperfusion. We investigated the effect of recombinant human angiopoietin-like protein 4 on intestinal barrier structure and function deterioration using intestinal ischemia/reperfusion models in rats as well as hypoxia/reoxygenation models in cells. Intestinal barrier indicators associated with the inflammatory and oxidative cascade, excessive activation of cellular autophagy and apoptosis were compared. Our results indicated that recombinant human angiopoietin-like protein 4 behaved as a promising therapeutic agent for intestinal ischemia/reperfusion-induced intestine injury.

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INTRODUCTION

Intestinal barrier dysfunction induced by ischemia/reperfusion (I/R) and atopic dermatitis is frequently observed in clinical practice. Hospital mortality due to acute mesenteric ischemia remains high, ranging from 60% to 90% over the past few decades [1]. Reperfusion following intestinal ischemia promotes extensive activation of inflam-

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matory response and oxidative stress, autophagy and apoptosis, leading to a dreadful illness, such as multiple organ dysfunction syndromes (MODS), especially breakdown of the intestinal barrier[2,3]. Recent studies have indicated that coronavirus disease 2019 associated with intestinal hypoperfusion may further deteriorate barrier dysfunction and acute lung injury *via* a positive feedback mechanism[4]. Although remarkable progress has been made, there is no widely accepted therapy to prevent I/R associated intestinal barrier dysfunction.

Integrity of the intestinal mucosal barrier is retained by the critical factors of tight junctions and intercellular junctions, including transmembrane proteins such as zonula occludens (ZO), claudins (CLDN) and junctional adhesion molecule. These consist of a complex that forms a selectively permeable seal between adjacent epithelial cells, which is a key element in the paradigm of gut-origin MODS[5,6]. Myosin light chain (MLC) phosphorylation *via* MLC kinase (MLCK) is critical in pathophysiological modulation of intestinal mucosal barrier breakdown. ZO-1 and CLDN-2 behaved specifically as loss and redistribution in intestinal mucosal barrier breakdown and may be regulated *via* an MLCK-dependent MLC phosphorylation signaling pathway[6-8]. Another critical factor is vascular leakage, which contributes to the intestinal barrier dysfunction after I/R. Microvascular endothelial cell layer including VE-cadherin (VE-cad) creates a semi-permeable barrier between tissue and blood for the transport of lipids, proteins and electrolytes. Microvascular barrier dysfunction is crucial in the initiation and progression of vascular barrier disruption after I/R[5,9,10].

Angiopoietin-like 4 (ANGPTL4) was identified by three independent research groups simultaneously and named ANGPTL4 by The HUGO Gene Nomenclature Committee in 2000[11]. Extensive investigations have been carried out over the past decades and found that this molecule is a remarkably dynamic entity that participates in a variety of pathophysiological conditions. The functions of ANGPTL4 have been involved in energy homoeostasis, wound healing, anoikis resistance, retinopathy angiogenesis and corneal epithelium repair as well as ischemic and barrier regulation [12]. Recent studies have reported that ANGPTL4 is a therapeutic agent in vasculoprotection and counteracting permeability of the blood-brain barrier in ischemic stroke [13]. Others also reported that ANGPTL4 blocked reactive oxygen species (ROS)-induced anoikis in cholangiocarcinoma cells[14]. It is postulated that ANGPTL4 may play a role in intestinal barrier integrity after intestinal I/R.

Here, we show that treatment with recombinant human ANGPTL4 (rhANGPTL4), in a rat model of intestinal I/R and intestinal epithelial (Caco-2) cells and human umbilical vein endothelial cells (HUVECs) with hypoxia/reoxygenation (H/R) *in vitro*, improves intestinal histopathology injury and attenuates the intestinal mucosa inflammatory and oxidant response. rhANGPTL4 counteracts the autophagy and apoptosis of the intestine and consequently diminishes the increase of permeability and loss of tight junction proteins of the intestine. Similar effects were also observed in intestinal epithelial cells and vascular endothelial cells. We provide the first empirical evidence that ANGPTL4 protects intestinal mucosa barrier integrity and confers intestine protection. ANGPTL4 might, therefore, constitute a relevant novel therapeutic approach for intestinal mucosa after I/R.

MATERIALS AND METHODS

Animals and ethics statement

Male Wistar rats weighing 180-220 g were purchased from Dalian Medical University Animal Experimental Center (Dalian, China, institutional protocol number: SCXK 2008-0002) and preserved under qualified experimental conditions with laboratory water and food. They were accommodated with an equipment shelter and kept at 25 °C and humidity of 40%-50% with a 12 h light-12 h dark cycle. Rats were acclimatized for 1 wk prior to experimentation. All steps were executed in line with the institutional animal care guiding principles and approved by the Institutional Ethics Committee.

Intestinal ischemia and reperfusion experiment

Intestinal I/R models were established according to the methods reported previously [15]. In brief, superior mesenteric artery was isolated following laparotomy and blocked softly using a non-invasive microvascular clip for 60 min. Occlusion was affirmed while the vessel beats paused and intestinal color faded, then clips were removed, and the procedure lasted 240 min. Sham animals received laparotomy and superior mesenteric artery identification but without clamping.

Animal treatment

Animals were randomly divided into 3 groups with 8 rats in each: (1) sham group; (2) vehicle (normal saline) + I/R group; and (3) I/R + rhANGPTL4 group. In group 3, rats were treated (*via* intravenous injection through tail vein) with rhANGPTL4 (28 mg/kg body weight in 0.5 mL normal saline) at the beginning of reperfusion. Rats in sham and I/R groups received normal saline with the same volume. All rats were sacrificed, and samples were collected for further investigation.

Collection of specimens

Blood samples of 5-6 mL were collected from the abdominal aorta at the end of reperfusion. They were preserved for 30 min at room temperature and centrifuged at 4 °C (3000 rpm for 15 min). The isolated serum was frozen at -80 °C. The supernatant was placed in a sterilized Eppendorf tube (Eppendorf Company, Limited, Shanghai, China) at -80 °C. Three centimeter proximal jejunum and distal ileum was separated after animals were sacrificed and then placed at -80 °C for further investigation.

Assessment of intestinal permeability

Intestinal mucosal barrier function was assessed by mucosal-to-serosal clearance of fluorescein isothiocyanate-conjugated dextran (4 kilodaltons; FD-4) in everted ileal sacs incubated *ex vivo* as previously described[16]. Intestinal microvascular permeability was investigated using VE-cad as the indicator.

Serum lactate dehydrogenase assay

Serum lactate dehydrogenase (LDH) was determined by a kinetic assay as previously described[16].

Histological examination

The jejunum segments were collected and embedded in 4% paraformaldehyde for histological evaluation. Tissues were dehydrated in graded ethanol, paraffin-embedded and sliced. Slices were prepared and stained using hematoxylin-eosin. The intestinal mucosal injury was assessed using a light microscope following the criteria described by Chiu *et al*[17].

TUNEL staining

TUNEL staining was performed following the manufacturer's instructions (In Situ Cell Death Detection Kit; Roche, Germany) and previously described method[18]. TUNEL positive cells were counted under a microscope (× 400), and the apoptotic cells in each slice were counted in seven independent areas followed by averaging.

Cell culture and H/R incubation

Caco-2 cells were acquired from the American Type Culture Collection (ATCC, Rockville, MD, United States). Cells were cultured in a humidified atmosphere of 5% CO_2 at 37 °C in Dulbecco's Modified Eagle Medium added with 1% nonessential amino acids, 10% fetal bovine serum and 1% glutamide (Gibco, CA, United States). In order to mimic intestinal I/R *in vivo*, Caco-2 cells were subjected to hypoxia for 1 h and reoxygenation for 4 h on passages 25–35 using microaerophilic equipment (Thermo Fisher Scientific, United States). Both small interfering RNA (siRNA) control and siRNA ANGPTL4 cells treated with H/R and vehicle were included. After H/R, supernatants were obtained from the monolayers and centrifuged at 1300 rpm for 10 min at 4 °C. The apoptosis of Caco-2 monolayers was determined instantly. Supernatants were preserved at -80 °C for further enzyme-linked immunosorbent assay. HUVECs (Lonza; passages 3–6) were seeded and grown in endothelial growth medium (EGM bullet kit; Thermo fisher, Shanghai, China) containing 10% (vol/vol) fetal bovine serum at 37 °C. H/R was performed as that for intestinal epithelium cells.

Transient transfection of siRNA

For siRNA transfection, 1 × 10^5 Caco-2 cells were seeded on six-well plates and transfected at the time of 70%-80% confluence with an ANGPTL4 siRNA or non-binding control siRNA using Lipofectamin 2000 (Invitrogen, Karlsru-he, Germany): siRNA ANGPTL4 sense 5′-AAAGCTGCAAGATGACCTCAGATGGAGGCTG-3′; anti-sense 5′-AAAAGGCTTAAGAAGGGAATCTTCTGGAAGAC-3′; siRNA control sense 5′-AAAGCTGTCTTCAAGATTGATATCGAAGACTA-3′; and anti-sense 5′-AAAATAGTCTTCGATATCAAGCTTGAAGAC A-3′. After 24 h incubation, the cells were washed, and fresh Dulbecco's Modified Eagle Medium containing 10% fetal

bovine serum was supplemented.

Expression and purification of rhANGPTL4 proteins

Full-length rhANGPTL4 was purchased from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel).

Measurement of intracellular reactive oxygen species ROS accumulation

Fluorescent probe DCFH-DA (Sigma Aldrich, United States) was used to observe the intracellular accumulation of ROS. After exchanging the proper medium, cells (5×10^5 cells/mL) were incubated in 10 μM DCFH-DA solution at 37 °C for 30 min and were examined using a confocal microscope outfitted with an argon laser (488 nm, 200 mW) as previously studied[19].

Detection of intestinal tissue and cell cytokines

Interleukin (IL)-6, tumor necrosis factor α (TNF-α) and IL-1β were quantified using rat enzyme-linked immunosorbent assay kits (BOSTER Bioengineering Co. Ltd., Wuhan, China) in intestinal tissues. Intestinal tissues were collected and coagulated on ice for 2 h. Intestinal samples were acquired and homogenized using a tissue homogenizer (IKA T10 Basic, Staufen, Germany) in 1 mL lysis buffer containing 150 mmol/L NaCl, 0.5% Triton × 100, 1 mmol/L $CaCl_2$, 15 mmol/L Tris and 1 mmol/L $MgCl_2$, pH 7.4. Homogenates were centrifuged at 10000 × *g* for 10 min. Supernatants were preserved at -80 °C for further investigation.

Intestinal epithelial cells were stimulated with H/R *in vitro* and treated with or without rhANGPTL4 as described above. Supernatants of cell homogenates were harvested after treatment and transferred to a -80 °C refrigerator for further analysis. TNF-α, IL-6 and IL-1β of the cell homogenate supernatants were determined using the enzyme-linked immunosorbent assay (Bender Med Systems, San Diego, CA, United States) and quantified according to the standard curve method.

Western blotting analysis

For detecting ANGPTL4, MLCK, phosphorylated MLC (p-MLC), MLC, ZO-1, CLDN-2, VE-cad, microtubule-associated protein light chain 3 (LC3)-I, LC3-II, p62, beclin-1 and caspase-3 content in the intestine and Caco-2 cells, 30 μg protein was extracted using 12% and 15% SDS-PAGE (Bio-Rad, Hercules, CA, United States), and moved to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, United States). Membranes were incubated with primary anti-ANGPTL4 antibody (Abcam Ltd., Cambridge, United Kingdom), anti-MLCK antibody (Abcam Ltd., Hong Kong, United Kingdom), p-MLC and MLC antibody (Cell Signaling Technology, Beverly, MA, United States), anti-ZO-1 and CLDN-2 antibody and anti-VE-cad (Santa Cruz Biotechnology, United States), anti-LC3-I, anti-LC3-II, anti-p62, anti-Beclin-1 antibody (Santa Cruz Biotechnology, United States), anti-caspase-3 and anti-cleaved-caspase-3 antibody (Bioworld, Minneapolis, MN, United States) or anti-β-actin antibody in Tris-phosphate-buffered solution overnight at 4 °C in 5% skimmed milk. The membranes were co-incubated with biotinylated secondary antibody and diluted 1:1000 in PBST containing 5% skimmed milk at 37 °C for 2 h after washing 3 times in TBS containing 0.1% Tween 20. Then membranes underwent large-scale elution with TBS containing 0.1% Tween and exposed using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology, Hangzhou, China).

Quantitative real-time polymerase chain reaction

Total RNA was collected from animals with Trizol kits (Invitrogen, Carlsbad, CA, United States) in line with the manufacturer's instructions. Reverse transcription into complementary DNA was executed using a TaKaRa RNA polymerase chain reaction (PCR) Kit (avian myeloblastosis virus) Version 3.0 (TaKaRa, Dalian, China) for PCR analysis; primers were described as follows: ANGPTL4 sense: 5′- AGACCCGAAG-GATAGAGTCCC-3′ (forward), antisense: 5′-CCTTCTGGAACAGTTGCTGG-3′ (reverse); β-actin sense: 5′-AGAGGGAAATCGTGCGTGAC-3′ (forward), antisense: 5′-CAATAGTGATGACCTGGCCGT-3′ (reverse). SYBR Premix Ex Taq kit (Takara, China) was used to perform quantitative real-time PCR, and ABI 7500 Fast Real-Time PCR System (Applied Biosystems) was used for fluorescence determination during amplification. PCR cycling was performed as follows: Initial annealing at 95 for 30 s, and 40 cycles at 95 °C for 5 s, 60 °C for 34 s and 72 °C for 30 s.

Statistical analysis

All measurements were calculated using mean ± SD. A one-way analysis of variance was used to compare the average values of all groups. The Student-Newman-Keuls/least significant difference test was used to compare the mean of all pairs. Student's *t* test was used for the comparison of two groups. Kaplan-Meier log-rank test was applied to analyze the survival. A *P* value less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 18.0 statistical software package (SPSS, Chicago, IL, United States).

RESULTS

Intestinal I/R induces endogenous ANGPTL4 expression

It has been reported that ANGPTL4 is induced in a mouse model of ischemic stroke and severe intestinal inflammation. We wondered whether ANGPTL4 might be expressed in the intestine after I/R in rats. Intestinal I/R injury markedly promoted the mRNA and protein expression of endogenous ANGPTL4 ($P < 0.05$; Figure 1). These findings indicate that endogenous ANGPTL4 is involved in intestinal I/R. Having shown that ANGPTL4 is expressed upon intestinal I/R, we then sought to investigate its effect on intestinal I/R.

rhANGPTL4 improves intestinal histopathologic injury and decreases LDH level after intestinal I/R

To illustrate the effect of extrinsic administration of ANGPTL4 on intestinal I/R injury, rhANGPTL4 (28 mg/kg body weight) was administered *via* the tail vein at the initiation of reperfusion. Intestinal I/R caused generalized and macroscopic necrosis with severe intestinal mucosal injury in intestinal areas paratactic to the macroscopically ischemic intestine ($P < 0.01$; Figure 2A). Similarly, serum LDH, a biochemical marker of tissue damage, was significantly elevated *vs* the sham group ($P < 0.01$; Figure 2B), suggesting the generalized tissue injury induced during this pathological process. Administration of rhANGPTL4 alleviated intestinal I/R injury significantly. Histopathologically, most parts of the intestine were free of secondary mucosal injury after treatment ($P < 0.01$; Figure 2A). Furthermore, LDH levels in rhANGPTL4-treated rats were significantly lower than in the sham group after I/R ($P < 0.01$; Figure 2B). This indicates that ANGPTL4 treatment alleviates the severity of reperfusion.

rhANGPTL4 regulates the intestinal permeability barrier and MLCK-tight junction pathway-related proteins in vivo

To elucidate the role of rhANGPTL4 in intestinal barrier structure and function, we evaluated intestinal permeability of FD-4, MLCK, p-MLC/MLC, ZO-1 and CLDN-2 protein expression in intestinal mucosa, and the well-established and the recently confirmed methods were used for evaluation of intestinal barrier function. In vehicle-treated animals, I/R caused obvious destruction of intestinal barrier function manifested as increased FD-4 clearance *vs* the sham group ($P < 0.01$; Figure 3A). Meanwhile, MLCK and p-MLC/total MLC were also increased coupled with a decrease of ZO-1 and CLDN-2 in vehicle-treated I/R animals. rhANGPTL4 treatment resulted in marked recovery of FD-4 clearance and changes of MLCK, p-MLC/total MLC, ZO-1 and CLDN-2 protein levels 4 h after I/R, close to normal levels compared with vehicle animals ($P < 0.01$; Figure 3B). This indicates that rhANGPTL4 administration improves both structural and functional repair of the intestinal barrier dysfunction induced by I/R. In addition, ANGPTL4 is involved in the changes of intestinal I/R-induced mucosal barrier structure (MLCK-tight junction protein signaling pathway) and function (FD4 clearance rate in intestinal permeability). rhANGPTL4 confers intestinal mucosal barrier protection by altering the structure and function of the intestinal mucosal barrier. In this process, the MLCK-regulated tight junction signaling pathway may be a potential mechanism. We further investigated the possible mechanism that is involved in the process of intestinal mucosa barrier function regulation.

rhANGPTL4 suppresses the intestinal inflammatory response and oxidative stress after intestinal I/R

Proinflammatory cytokines are main contributing factors for the disruption of intestinal barrier dysfunction after I/R. We evaluated whether the intestinal cytokines

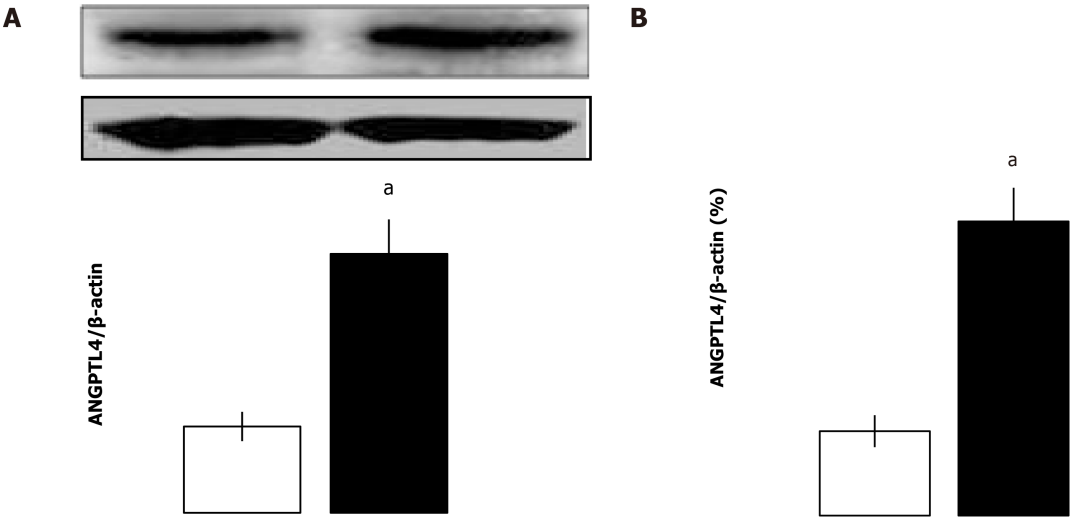


Figure 1 Intestinal angiopoietin-like protein 4 level after intestinal ischemia-reperfusion in different groups (mean ± SD, *n* = 8). A: Angiopoietin-like protein 4 protein levels were assessed by western blot; B: Angiopoietin-like protein 4 mRNA levels in the intestine were measured by quantitative real-time polymerase chain reaction. [a]*P* < 0.05 *vs* sham by Student's *t* test. ANGPTL4: Angiopoietin-like protein 4.

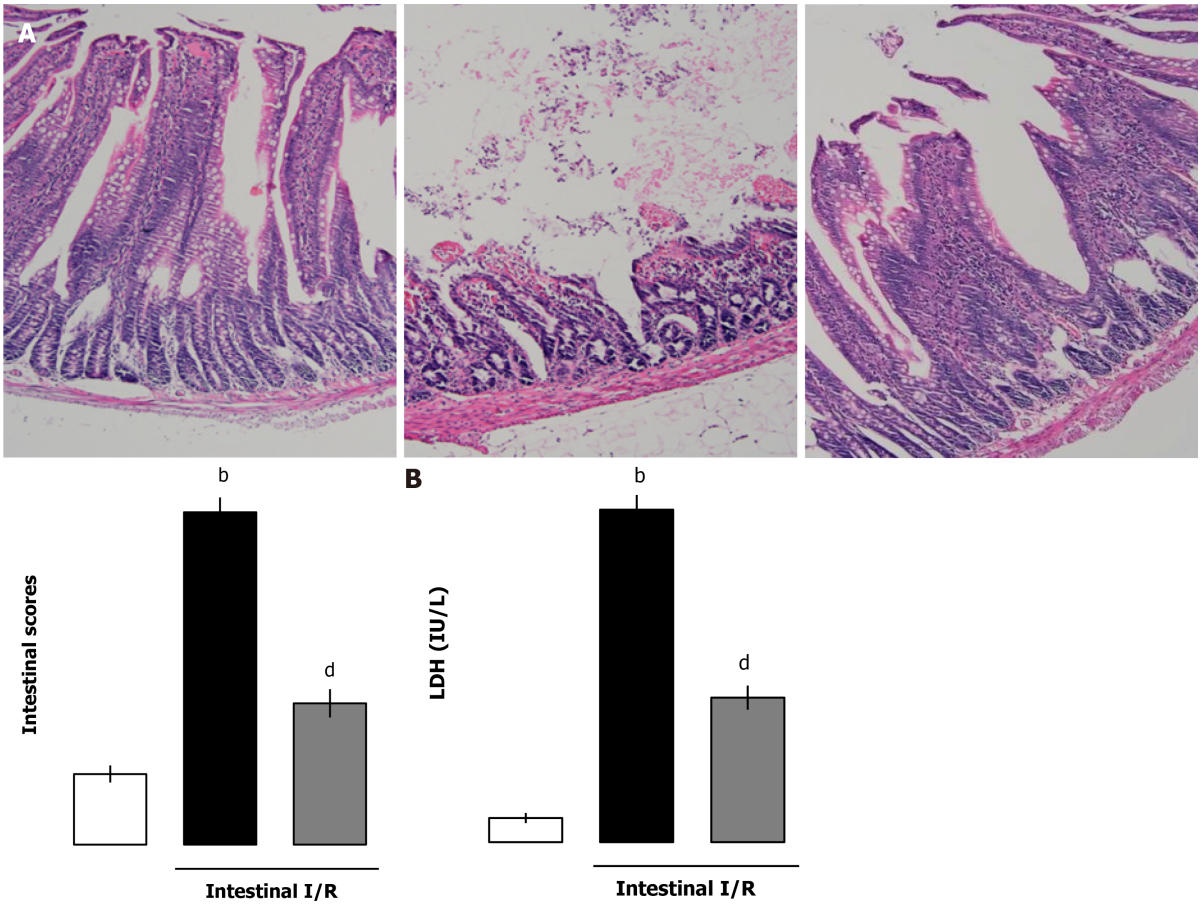


Figure 2 Recombinant human angiopoietin-like protein 4 improved ischemia-reperfusion-induced intestinal histopathologically and serum lactate dehydrogenase. A and B: Pathologic changes of intestine (scale bar = 100 μm) of (A) tissue and (B) serum lactate dehydrogenase (LDH) in different groups (mean ± SD, *n* = 8). [b]*P* < 0.01 *vs* sham; [d]*P* < 0.01 *vs* ischemia-reperfusion. I/R: Ischemia-reperfusion.

TNF-α, IL-1β, IL-6 and myeloperoxidase (MPO) levels were affected by administration of rhANGPTL4. Although the cytokines and MPO increased markedly after I/R, rhANGPTL4 dramatically reduced the proinflammatory response ($P < 0.01$, $P < 0.05$; Figure 4A-C) and MPO level ($P < 0.05$; Figure 4D). To investigate whether rhANGPTL4 influences oxidative damage to tissues, we further determined intestinal

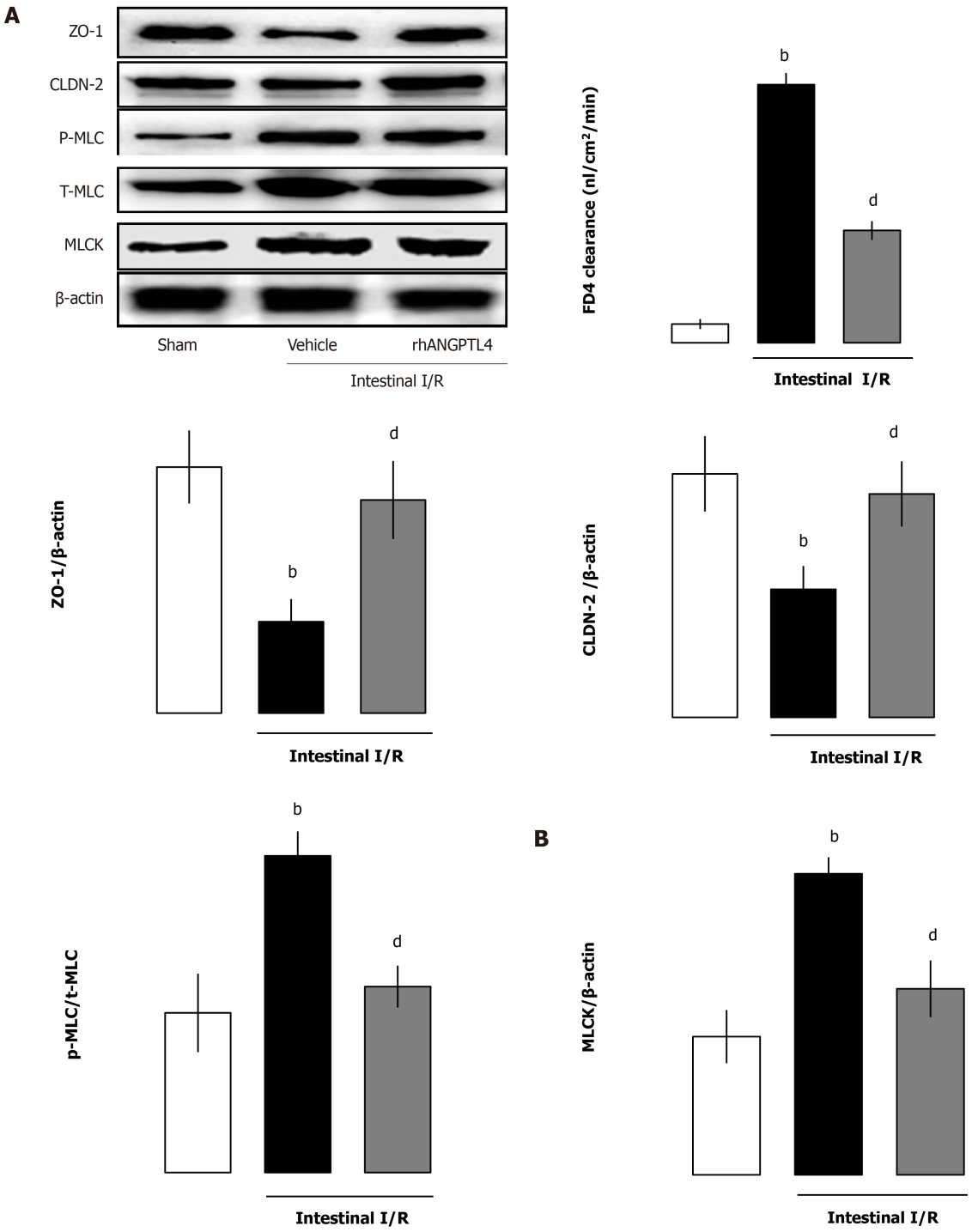


Figure 3 Recombinant human angiopoietin-like protein 4 regulates the intestinal permeability barrier and myosin light chain kinase-tight junction pathway related proteins *in vivo*. Intestinal mucosal tissue myosin light chain kinase (MLCK), phosphorylated-myosin light chain (p-MLC)/myosin light chain, zonula occludens-1 (ZO-1) and claudin-2 (CLDN-2) (A) and intestinal mucosal permeability (B) in different groups (mean ± SD, $n = 8$). $^{b}P < 0.01$ *vs* sham; $^{d}P < 0.01$ *vs* ischemia-reperfusion (I/R). ANGPTL4: Angiopoietin-like protein 4; t-MLC: Total-myosin light chain.

malondialdehyde (MDA). Intestinal MDA levels reduced significantly after rhANGPTL4 treatment ($P < 0.05$; Figure 4E). Changes of these indicators were associated with intestinal barrier dysfunction. This suggests that inflammatory cytokines and the oxidative stress cascade are contributing factors in destructed intestinal barrier.

rhANGPTL4 reduces the increase of intestinal autophagy and apoptosis after I/R

Intestinal I/R increased intestinal autophagy-related protein levels (beclin-1, LC3-I and LC3-II) but decreased the substrate p62 protein levels ($P < 0.05$; Figure 5A). As a classical apoptotic marker, the cleaved caspase-3 protein expression and numbers of

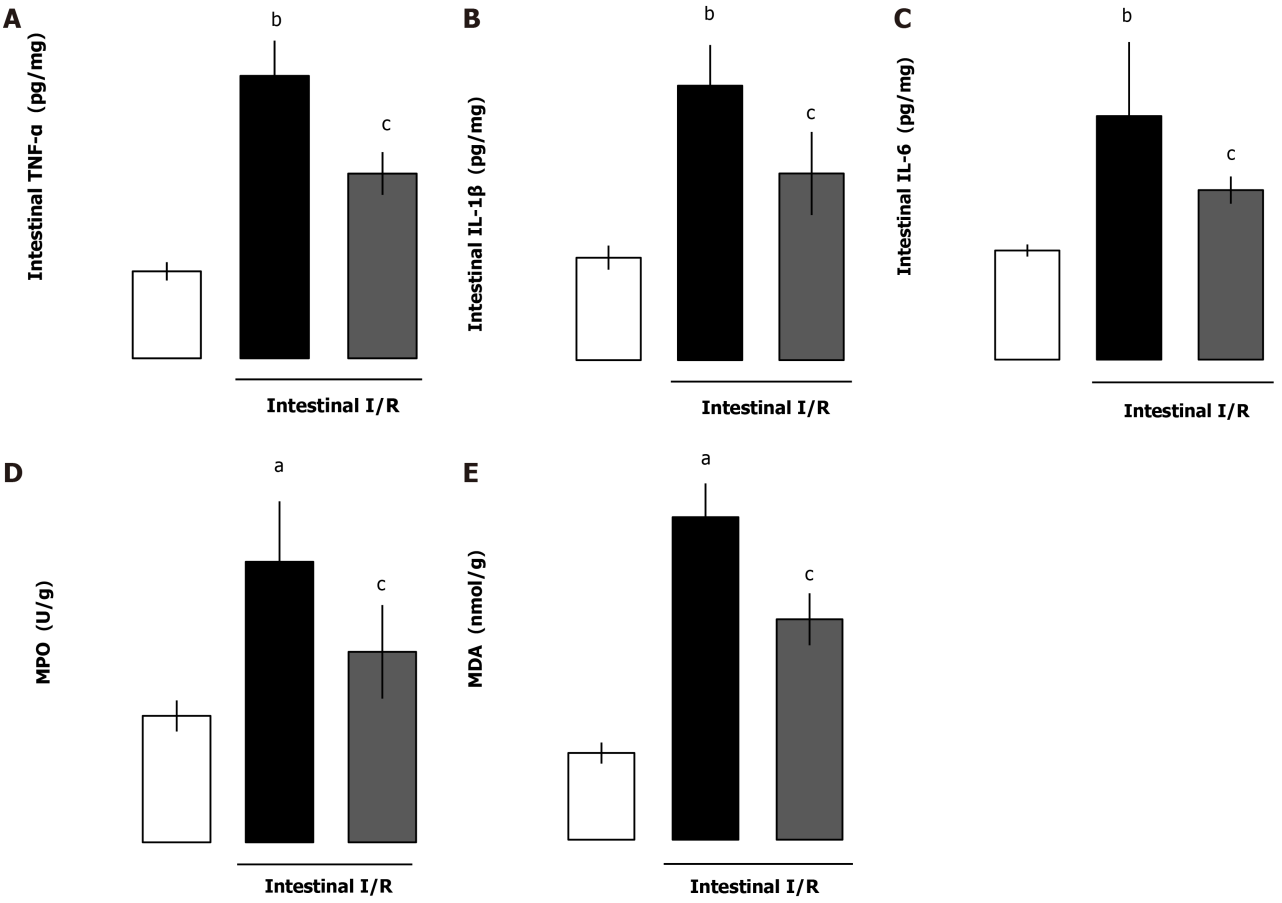


Figure 4 Recombinant human angiopoietin-like protein 4 suppresses the intestinal inflammatory response and oxidation stress after intestinal ischemia-reperfusion. A-E: Intestinal cytokine tumor necrosis factor-alpha (TNF-α) (A), interleukin (IL)-1β (B), interleukin-6 (C), intestinal myeloperoxidase (MPO) (D) and malondialdehyde (MDA) (E) levels in different groups (mean ± SD, n = 8). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* sham; $^{c}P < 0.05$ *vs* ischemia-reperfusion (I/R).

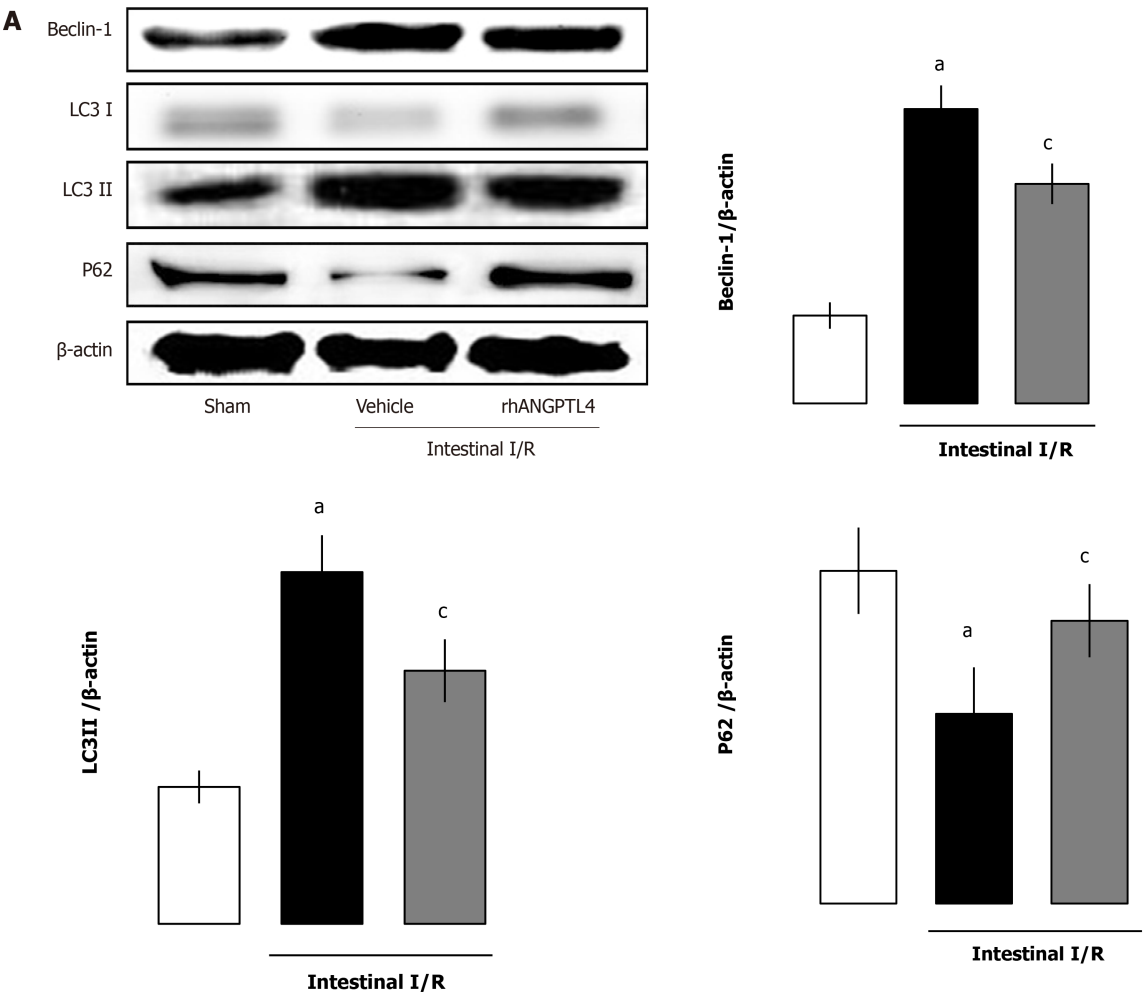
apoptotic cells by TUNEL staining in the intestine was markedly increased after I/R. Treatment with rhANGPTL4 decreased intestinal cleaved caspase-3 levels and apoptotic cell numbers remarkably to those in the sham group ($P < 0.05$; Figure 5B and 5C). At the same time, we found that the autophagy–related proteins (beclin-1, LC3-I, and LC3-II) and the substrate p62 in the intestine also recovered after treatment with rhANGPTL4 to those in sham animals ($P < 0.05$; Figure 5A). This indicates that excessive activated autophagy and apoptosis are crucial in the destruction of intestinal barrier integrity, and rhANGPTL4 is an effective agent to inhibit autophagy and apoptosis in intestinal I/R.

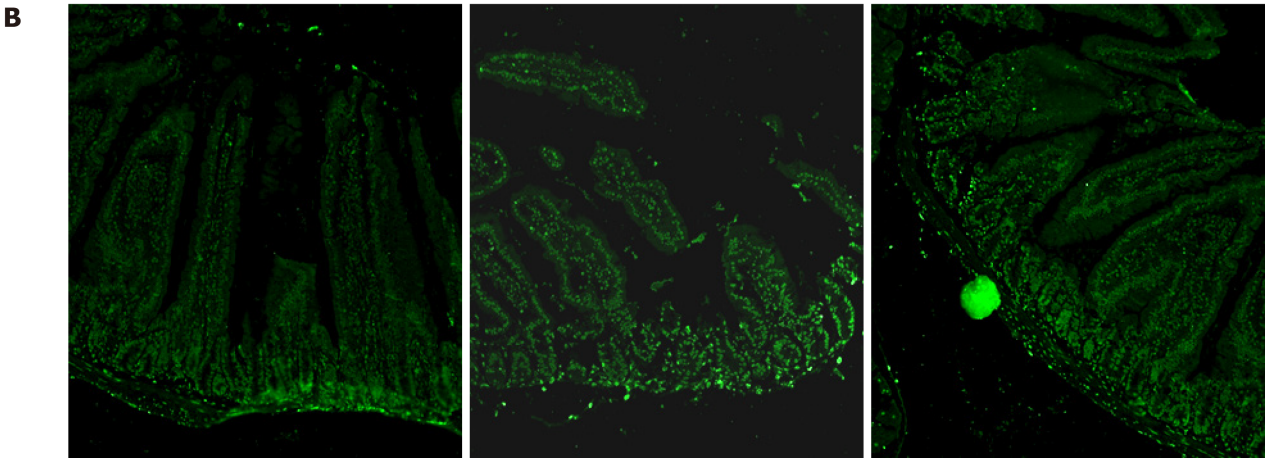
rhANGPTL4 improves animal survival after intestinal I/R

Animals in the vehicle group were sacrificed < 24 h following intestinal I/R injury ($P < 0.05$; Figure 6). However, rhANGPTL4-treated rats exhibited significant longer survival. The 24 h survival rate of rhANGPTL4 group was increased markedly compared to vehicle animals.

ANGPTL4 deficiency aggravates intestinal epithelial cell injury after H/R in vitro

To illustrate the effect of ANGPTL4 in intestinal I/R-induced barrier dysfunction, intestinal epithelial cells were used and siANGPTL4 was challenged by H/R to imitate intestinal I/R. We investigated the inflammatory response, ROS level, autophagy and apoptosis, and intestinal barrier function was measured using the MLCK-tight junction pathway-related protein in the intestinal epithelial cells. Compared with the vehicle group TNF-α and IL-6 levels associated with ROS levels upregulated significantly in both the siANGPTL4 control and siANGPTL4 groups after H/R. The same trends of LC3-II and cleaved caspase-3 but contrary expression of ZO-1 and CLDN-2 were observed correspondingly ($P < 0.01$; Figure 7). These indicate that ANGPTL4 is essential for maintaining the integrity of intestinal epithelial cell barrier function when challenged by H/R.


A
Beclin-1
LC3 I
LC3 II
P62
β-actin
Sham
Vehicle
rhANGPTL4
Intestinal I/R
Beclin-1/β-actin
a
c
Intestinal I/R
LC3II /β-actin
a
c
Intestinal I/R
P62 /β-actin
a
c
Intestinal I/R


B

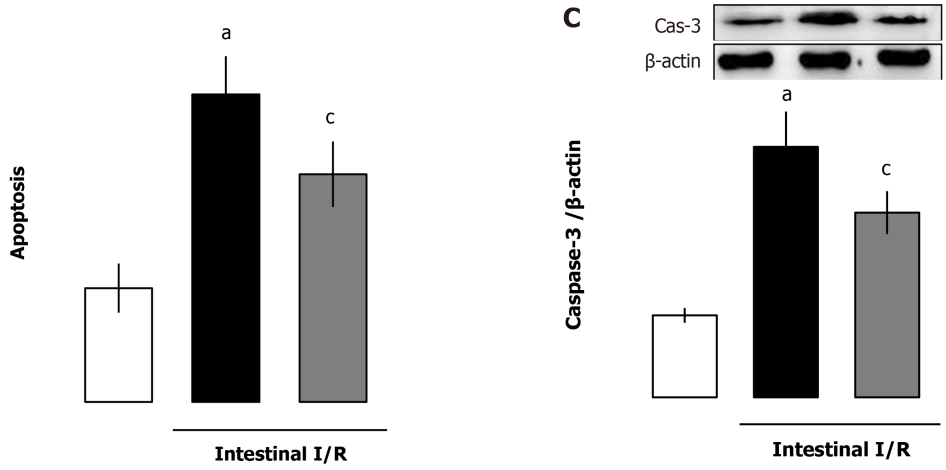


Figure 5 Recombinant human angiopoietin-like protein 4 reduced the increase of intestinal autophagy and apoptosis after ischemia-reperfusion. A: Intestinal autophagy-related protein beclin-1, microtubule-associated light chain 3 (LC3) I/II and p62 in different groups (mean ± SD, n = 8). ^{a}P < 0.05 *vs* sham; ^{c}P < 0.05 *vs* ischemia-reperfusion (I/R) groups; B and C: Intestinal apoptosis (scale bar = 100 μm) (B) and cleaved caspase-3 (Cas-3) (C) in different groups (mean ± SD, n = 8). ^{a}P < 0.05 *vs* sham; ^{c}P < 0.05 *vs* I/R. ANGPTL4: Angiopoietin-like protein 4.

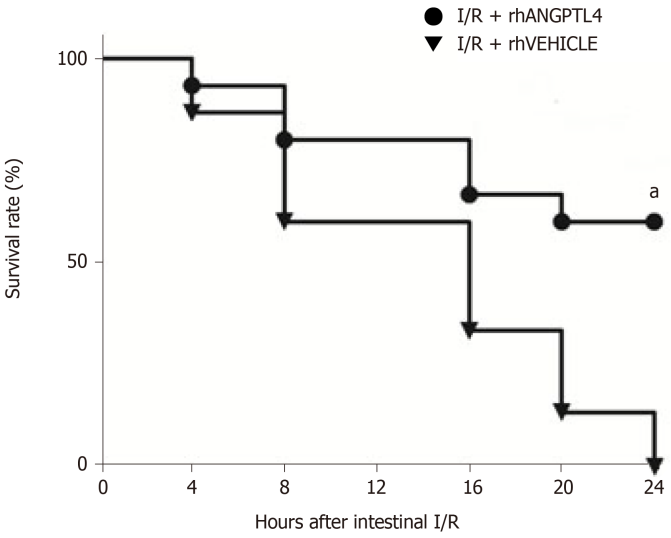


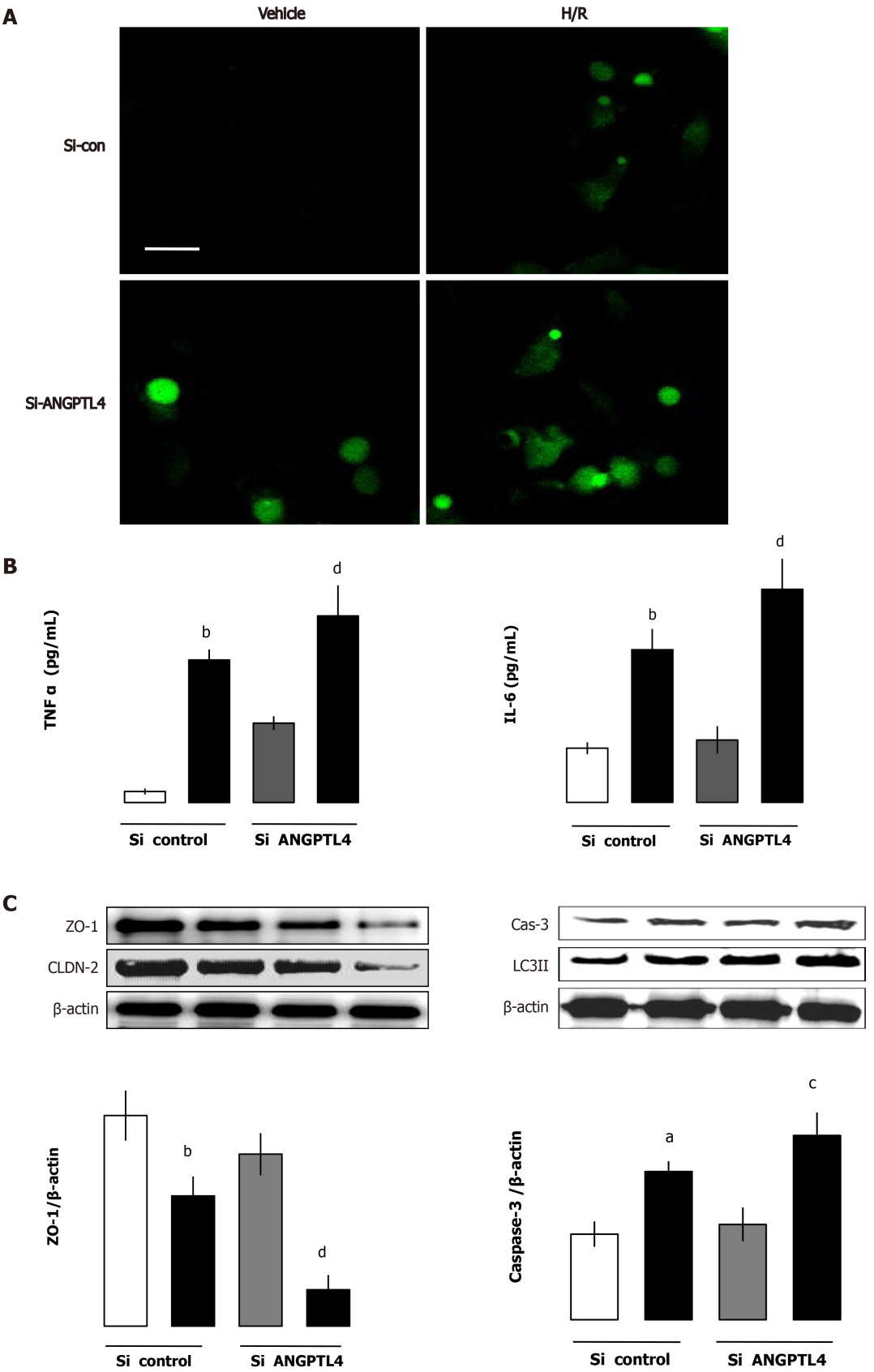
Figure 6 Survival benefit in recombinant human angiopoietin-like protein 4-treated rats after intestinal ischemia-reperfusion injury (n = 15, Kaplan Meyer log-rank test). Each point in the figure shows the mean survival rate at each time point. ^{a}P < 0.05 *vs* vehicle. I/R: Ischemia-reperfusion; rhANGPTL4: Recombinant human angiopoietin-like protein 4.

Administration of rhANGPTL4 protects against intestinal epithelial cell injury after H/R in vitro

To assess the effect of rhANGPTL4 on injury induced by H/R in siRNA ANGPTL4 (siANGPTL4) Caco-2 cells, rhANGPTL4 (10 μg/mL) or vehicle was injected before the H/R as previously described. Cell samples were harvested after reoxygenation for 4 h. Proinflammatory cytokines (including TNF-α and IL-6), MDA, cleaved caspase-3 and LC3-II levels and intestinal barrier-regulated protein expression of MLCK, ZO-1 and CLDN-2 were detected. Administration of rhANGPTL4 decreased proinflammatory cytokine (TNF-α and IL-6) levels and oxidative damage (MDA levels), inhibited autophagy (LC3-II levels) and apoptosis (cleaved caspase-3 levels) and reduced MLCK but restored the levels of ZO-1 and CLDN-2 after H/R in siANGPTL4 cells (P < 0.05, P < 0.01; Figure 8).

Administration of rhANGPTL4 protects against HUVECs injury after H/R in vitro

To investigate the role of rhANGPTL4 in vascular leakage induced by H/R in siANGPTL4 HUVECs, VE-cad was used as an indicator that plays a crucial role in the integrity of vascular endothelial function. rhANGPTL4 (10 μg/mL) or vehicle was


A
Vehicle
H/R
SI-con
SI-ANGPTL4
B
TNF α (pg/mL)
b
d
Si control
Si ANGPTL4
IL-6 (pg/mL)
b
d
Si control
Si ANGPTL4
C
ZO-1
CLDN-2
β-actin
Cas-3
LC3II
β-actin
ZO-1/β-actin
b
d
Si control
Si ANGPTL4
Caspase-3 /β-actin
a
c
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Si ANGPTL4

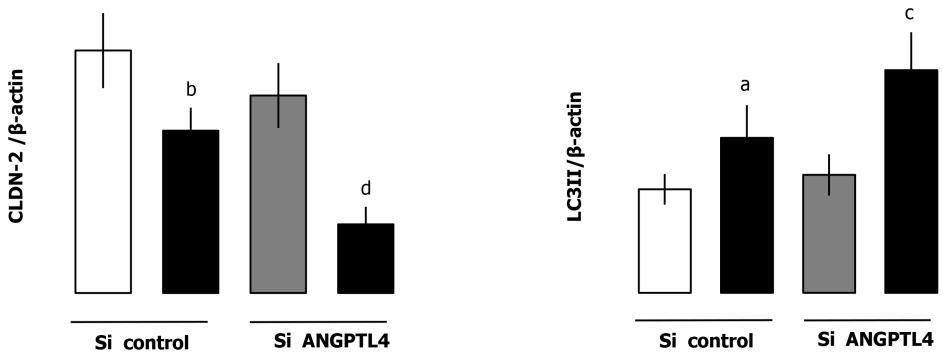


Figure 7 Angiopoietin-like protein 4 deficiency aggravates Caco-2 cells injury challenge by hypoxia/reoxygenation. A and B: Reactive oxygen species (ROS) levels (scale bar = 12.5 μm) (A) determined by immunofluorescence and tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6 levels (B) determined by enzyme-linked immunosorbent assay; C: Tight junction zonula occludens-1 (ZO-1) and claudin-2 (CLDN-2), autophagy marker microtubule-associated protein light chain 3 (LC3) II and apoptosis marker caspase-3 were determined using western blot assays (mean ± SD, $n = 3$). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* vehicle; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* vehicle ($n = 3$ per group). ANGPTL4: Angiopoietin-like protein 4; H/R: Hypoxia/reoxygenation; si: Small interfering RNA.

injected before H/R. Cell samples were collected at 4 h after reoxygenation. VE-cad protein expression was detected. Administration of rhANGPTL4 restored VE-cad protein expression levels significantly. Both ANGPTL4 vicious and ANGPTL4 control HUVECs exhibited low levels of VE-cad after H/R compared with vehicle cells ($P < 0.01$; Figure 9).

DISCUSSION

In the present study, we provide evidence and a potential mechanism that rhANGPTL4 alleviates intestinal I/R induced injury. *In vivo* rhANGPTL4 was found to (1) ablate intestinal tissue damage after I/R; (2) improve survival rate; (3) relieve the inflammatory response through inhibition of proinflammatory cytokines and restrict MPO, MDA and LDH; (4) lessen the autophagy and apoptosis molecules; and (5) maintain epithelial barrier function by restoration of tight junction (ZO-1 and CLDN2), inhibiting MLCK and phosphorylation of MLC and intestinal barrier permeability (FD-4 clearance rate). I*n vitro* studies showed that (1) ANGPTL4 deficiency aggravated the inflammatory and oxidative response, apoptosis and autophagy, associated with the loss of intestinal barrier tight junction; (2) rhANGPTL4 had the ability to inhibit inflammation and oxidation, autophagy and apoptosis and preserve the tighten junctions, and accordingly it maintained the endothelial barrier integrity under ANGPTL4 vicious circumstances; and (3) rhANGPTL4 inhibited vascular leakage *via* limiting the endothelial dysfunction (loss of VE-cad) in HUVECs challenged by H/R.

Intestinal I/R injury is accompanied by enhanced inflammatory cytokines and ROS cascade, augmented apoptosis and autophagy, vascular leakage and aggravated mucosal lesions[1-4,9]. Intestinal barrier breakdown has been implicated as an important turning point in intestinal I/R injury[7]. The intestinal barrier is the largest defender between the intestine and distant organs including lung and liver, which is critical in the development of MODS[13,18]. As a filter with selective permeability, this interface allows absorption of essential nutrients and eliminates the invasion of deteriorating products, including proinflammatory factors, ROS, *etc*. Exacerbating the inflammatory response and oxidative stress can induce intestinal epithelial cell apoptosis and autophagy, consequently destroying the intestinal mucosal barrier. In addition, intestinal mucosal breakdown facilitates the inflammatory mediators and ROS associated with the bacteria- derived products to translocate to extraintestinal organs[1-3,15,20]. Until now, the underlying mechanisms/factors of I/R inducing intestinal barrier breakdown are still unclear. Recent studies reported that MLC phosphorylation mediated by upregulated MLCK is crucial to intestinal barrier breakdown induced by hypoxia or proinflammatory cytokines[7]. Tight junctions ZO-1 and CLDN-2 regulated by the MLCK pathway are the two crucial factors regulating the intestinal barrier in I/R[5].

ANGPTL4 is normally secreted from the intestinal villus epithelium and modulated by the intestinal microbiota. Studies have shown that ANGPTL4 is essential to resist the loss of villus endothelial and lymphocyte populations to radiation-induced apoptosis[21]. As it is reported that ANGPTL4 is crucial in the postnatal lymphatic

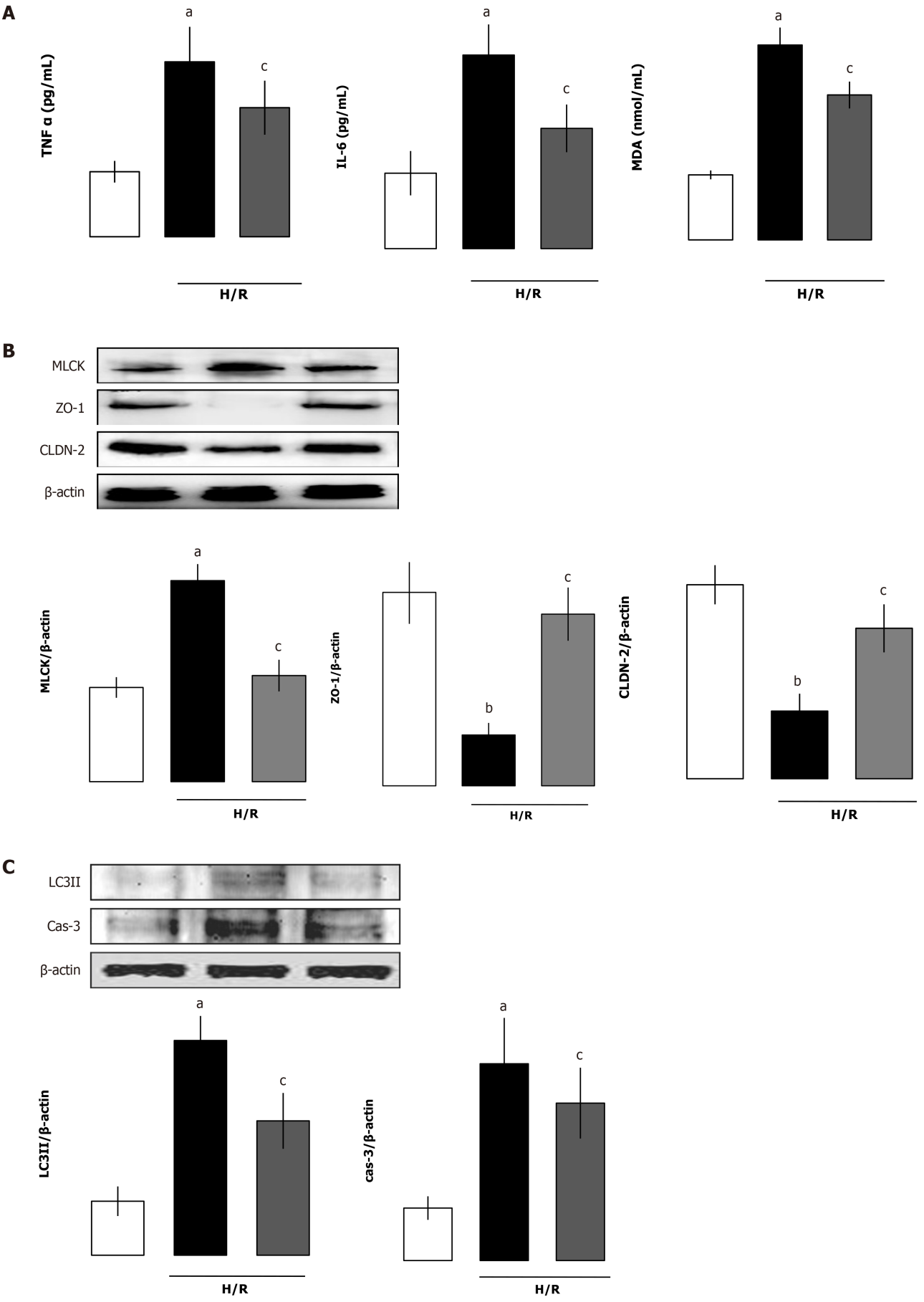


Figure 8 Beneficial effects of recombinant human angiopoietin-like protein 4 on angiopoietin-like protein 4 deficient Caco-2 cells challenged after hypoxia/reoxygenation. A: Cellular levels of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6 and malondialdehyde (MDA) levels; B:

Myosin light chain kinase (MLCK), zonula occludens-1 (ZO-1) and claudin-2 (CLDN-2) levels; C: Microtubule-associated protein light chain 3II and cleaved caspase-3 levels were measured 4 h after reoxygenation (mean ± SD, $n = 3$). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control; $^{c}P < 0.05$ *vs* vehicle. H/R: Hypoxia/reoxygenation.

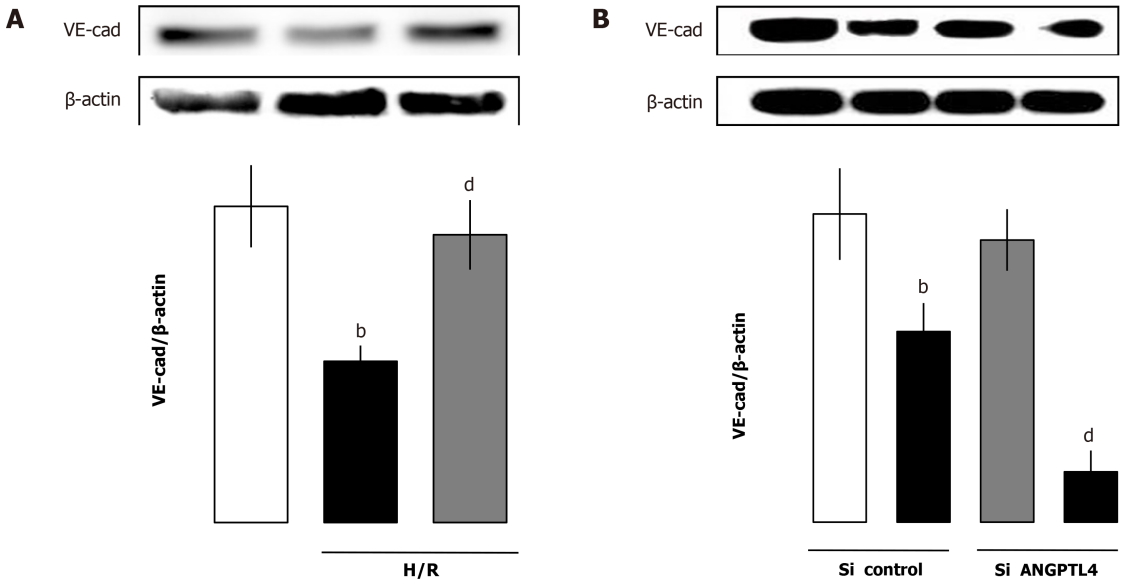


Figure 9 Levels of VE-cadherin after hypoxia/reoxygenation compared with respective vehicle cells. Recombinant human angiopoietin-like protein 4 restored the VE-cadherin (VE-cad) in human umbilical vein endothelial cells (A) after hypoxia/reoxygenation and VE-cadherin levels in small interfering (si) RNA angiopoietin-like protein 4 control and small interfering RNA angiopoietin-like protein 4 (ANGPLT4) human umbilical vein endothelial cells (B) of different groups (mean ± SD, $n = 3$). $^{b}P < 0.01$ *vs* vehicle; $^{d}P < 0.01$ *vs* vehicle. H/R: Hypoxia/reoxygenation.

partitioning and disease, it is not unexpected that mice missing ANGPTL4 may die during the suckling period with dilated intestinal lymphatic vessels[21,22]. It is noteworthy that injection of rhANGPTL4 *in vivo* blocked VEGF-driven separation of the VEGFR2/VE-cad complexes, reduced myocardial infarct area and the no-reflow degree in animals. ANGPTL4 exhibited a therapeutic vasculoprotection on no-reflow and exerted cardioprotection[23-26].

However, the role of ANGPTL4 in various types of cells has not been evaluated in detail. We investigated the effects of ANGPTL4 and rhANGPTL4 in maintaining the epithelial barrier through various mechanisms under basal and I/R (H/R) conditions. This preliminary analysis indicated an assumed effect of ANGPTL4 in the circumstance of intestinal I/R-associated endothelial and epithelium barrier damage. In this study, we demonstrate that after I/R injury, intestinal barrier breakdown is associated with the up-regulation of both MLCK pathway-related protein expression and permeability of intestinal mucosa. An associated loss of tight junction protein ZO-1 and CLDN-2 as well as increased histological damage of mucosa were observed following I/R. However, treatment with rhANGPTL4 not only blocked the upregulation of MLCK pathway- related protein and intestinal mucosal permeability but also restored the tight junction proteins ZO-1 and CLDN-2 associated with a lower intestinal histological damage of mucosa. Similar results were also observed in ANGPTL4-deficient intestinal epithelial cells when challenged by H/R. However, this was not observed in the intestinal epithelial vehicle cells without H/R. This indicates that the role of ANGPTL4 is silent in normal circumstances but essential in the cellular homeostasis upon H/R stress stimulation.

It is widely accepted that the oxidative stress and inflammation cascade may contribute to the intestinal barrier destruction through various mechanisms. Activated and influxed immune cells including neutrophils and macrophages will release amplified proinflammatory cytokines (including TNF-α, IL-6 and IL-1β) associated with oxygen free radicals. Higher levels of circulating proinflammatory cytokines and oxygen free radical activation lead to a loss of tight junction, eventually causing intestinal barrier breakdown induced by intestinal I/R[2,16]. Thus, a large number of studies have confirmed that the anti-inflammatory and antioxidative strategies are protective against breakdown of the barrier induced by I/R.

A previous study implicated that ANGPTL4 deficiency can aggravate the severe intestinal inflammation induced by high dietary saturated fat in rats[27]. Further studies showed that rhANGPTL4 protected ischemic stroke and exhibited antioxidative and anti-inflammatory effects[13]. We investigated the effect of ANGPTL4 on oxidative stress and inflammation during intestinal I/R. We found an enhanced expression of ANGPTL4 in rats with intestinal I/R and human intestinal epithelial cells stimulated with H/R, which indicated that ANGPTL4 exerted an effect on modulating the inflammatory milieu following intestinal I/R for triggering the progression of intestinal barrier dysfunction. Our results showed the antioxidative and anti-inflammatory effects of rhANGPTL4 on intestinal barrier dysfunction after I/R, and rhANGPTL4 alleviated the inflammatory and oxidative cascade both *in vivo* and *in vitro*. ANGPTL4-mediated barrier protection is due to, at least partially, the inhibition of the inflammatory and oxidative cascade.

In addition to the inflammatory and oxidative cascade, apoptosis and autophagy are mutual connection pathways, and both have direct disrupting roles in intestinal epithelial cells and the intestinal mucosal barrier in response to I/R[3,4,28]. An essential level of autophagy and apoptosis is important for maintaining intestinal epithelial cellular homeostasis under normal conditions. However, if the stress persists, autophagy will not afford cell surviving anymore. Instead exorbitant autophagy and copious activation of apoptosis will cause dramatic disruption of intestinal mucosa and barrier breakdown[28]. We first explored whether increased autophagy and apoptosis were related with ANGPTL4 in intestinal I/R of a rat model and the inhibition effects of rhANGPTL4 on autophagy and apoptosis both *in vivo* and *in vitro* challenged by I/R (H/R).

Autophagy is a lysosome-mediated degradative pathway of cellular mechanisms for degrading misfolded protein in addition to the ubiquitin-proteasome system of ubiquitinated proteins[29]. The implementation of autophagy involves a series of autophagy-related proteins that are essential for induction of autophagy and recycling of autophagosomes. Microtubule-associated protein LC3 exists in two forms including LC3-I and its proteolytic derivative LC3-II. LC3-1 is conjugated with phosphatidylethanolamine to become LC3-II, which can be used as a hallmark of autophagy because its population correlates with the number of autophagosomes. P62/SQSTM1 is selectively incorporated into autophagosomes through binding to LC3 and is affirmed as a specific substrate that degrades *via* the autophagy-lysosomal pathway. Therefore, the p62 contents are oppositely parallel with autophagic activity. Beclin 1 (ATG6) is the first identified mammalian gene to mediate autophagy and is implicated in the nucleation of the autophagic vesicle, which is another marker of autophagy[30].

Recent studies have shown that excessive autophagy that is active in necrotizing enterocolitis and inhibiting autophagy is protective against focal cerebral ischemia in rats[4,31]. Intestinal mucosal barrier disruption is associated with excessive autophagy activation. Our results suggested that ANGPTL4 conferred intestinal mucosal barrier preservation and inhibited the excessive activated autophagy in intestinal I/R. In the present study, intestinal autophagy activated massively in rats with intestinal I/R. Upregulated expression of required autophagy markers (beclin 1 and LC3-II) and the loss of p62 protein are found in intestinal epithelium both *in vivo* and *in vitro* challenged by I/R or H/R. Consequentially activated autophagy caused accumulation of autophagic vacuoles and disrupted the majority of the cytosol and organelles, eventually causing the collapse of vital cell functions and cell death. The attenuated epithelial cell homeostasis makes intestine mucosal injury more vulnerable upon I/R. However, rhANGPTL4 reversed I/R-induced changes of autophagy-related regulators (LC3-II, beclin 1 and p62) both *in vivo* and *in vitro*. This suggested that ANGPTL4 limited intestinal mucosal exorbitant autophagy to maintain the integrity of intestinal barrier.

Apoptosis was demonstrated to be the main type of cell death in intestinal I/R. Apoptosis disrupts the epithelial cell layer directly when the intestine is subjected to I/R[4]. Studies have shown that "anoikis" exerts a crucial effect in inducing apoptosis during intestine I/R. "Anoikis" manifested as contact disrupted cell death occurring between extracellular matrix materials and cells[32]. Ikeda *et al*[3] reported that affected epithelial cells lost their link with the villous mesenchyma, and thus apoptosis induced by I/R could represent "anoikis". They also revealed that distribution of epithelial cell-matrix interactions ("anoikis") exhibited a crucial effect on initiating apoptosis in detached enterocytes[4]. It is worth noting that ANGPTL4 has been demonstrated to promote anoikis resistance in cholangiocarcinoma and hepatoma cells grown in a disassociated status. These cells try to shape a synoikis-like multi-cellular assemblage that resists apoptosis occurrence. The inhibition of ANGPTL4 by RNA interference increased detachment-related apoptosis and made tumor cells sensitized

to antitumor drugs[14]. This implicates that intestinal epithelial cells may drive a similar mechanism to resist I/R-related apoptosis in intestinal mucosa. Our current results show that rhANGPTL4 resisted apoptosis and limited the increased levels of cleaved casepase-3 both *in vivo* and *in vitro*. Cells lack of ANGPTL4 showed increased apoptosis and cleaved casepase-3 levels when challenged by H/R. It can be concluded that ANGPTL4 may protect against intestinal mucosal barrier dysfunction due to I/R through anoikis resistance.

Another key factor for the intestinal barrier preservation that cannot be ignored during intestinal I/R is the vascular permeability[9]. Patients exhibited loss of plasma proteins and other macromolecules induced by increased permeability in the colonic microvasculature. During the perpetuation of intestinal I/R, microvascular injury is ubiquitously present. Intestinal microvessels manifested apparently as endothelial dysfunction. Microvascular endothelial cell layer consists of closely arranged ECs that shape a semi-permeable barrier between tissue and blood for the transport of lipids, proteins and electrolytes[33]. Therefore, microvascular barrier dysfunction exerts an important effect in the beginning and progressive phase of I/R injury. Consequently, endothelium reformation induced microvascular permeability changes and exerted a crucial effect on intestinal barrier dysfunction. The bidirectional regulation role of ANGPTL4 in vascular permeability has been extensively studied in various diseases [34-36]. However, the association of ANGPTL4 with vascular leakage induced by I/R remains obscure. In the present study, HUVECs with RNA interference and H/R exhibited a significant loss of the key regulator VE-cad. In addition, rhANGPTL4 inhibited the vascular permeability significantly, manifested as a change of VE-cad in HUVECs challenged by H/R. It is concluded that ANGPTL4 protects the intestinal barrier at least partially *via* inhibiting vascular leakage.

Intestinal I/R could still be an important cause of MODS, and it is of significance to note that besides the reduction of intestinal structure, ANGPTL4 treatment also significantly improved the intestinal barrier function.

CONCLUSION

This study demonstrates that rhANGPTL4 can lessen I/R injury by maintaining intestinal barrier structure and function. ANGPTL4 showed a conspicuous effect on MLCK-related proteins, tight junction and vascular permeability as well as on the inflammatory and oxidative response, autophagy and apoptosis. This implies that ANGPTL4 plays a significant role in modulating intestinal barrier integrity. It is speculated that rhANGPTL4 is important in intestinal barrier function maintenance and may have clinical advantages in I/R-related clinical circumstances. Further studies are needed to investigate the protective role of ANGPTL4 on remote organ dysfunction induced by intestinal I/R and to clarify whether this therapeutic agent targeting protection against the intestinal barrier dysfunction could be applied to patients in intensive care units.

ARTICLE HIGHLIGHTS

Research background

Intestinal barrier breakdown remains frequently complicated in critical care patients of intestinal ischemia-reperfusion (I/R), severe acute pancreatitis and sepsis. Although vigorous experiments are performed in this field, the application of an instant effective agent or therapy in clinical has not yet been discovered. Recombinant human angiopoietin-like protein 4 (rhANGPTL4) is known to be protective to the blood-brain barrier when administered exogenously, and endogenous ANGPTL4 deficiency deteriorates intestinal injury.

Research motivation

We intend to explore and discover a novel and promising agent to confer intestinal barrier protection induced by I/R. Our results indicated recombinant agents that exhibit intestinal barrier protective characteristics. The agents may be safer and facilitated to translate into clinical use.

Research objectives

Understanding and regulating ANGPTL4 as a therapeutic target and recombinant human ANGPTL4 will be an application in clinical use in patients suffering from intestinal barrier dysfunction.

Research methods

This research was executed using Wistar rats treated with intestinal I/R and intestinal epithelial (Caco-2) cells and human umbilical vein endothelial cells stimulated with H/R to intimate the I/R pathogenesis *in vivo*. Further, RNA interference was performed, and recombinant ANGPTL4 has been evaluated.

Research results

A loss of crypt epithelium and myocytes were observed in the muscularis propria. Intraluminal microdialysis were changed, as well as the biochemistry indicators were remarkably enhanced following intestinal I/R. Recombinant human ANGPTL4 treatment significantly reversed indicators above associated with inhibiting the inflammatory and oxidative cascade, excessive activation of cellular autophagy and apoptosis and was associated with an improvement of survival rate. *In vitro* studies showed similar results in Caco-2 and human umbilical vein endothelial cells.

Research conclusions

Recombinant human ANGPTL4 achieved an optimal therapeutic effect for intestinal I/R-induced intestinal barrier injury. Using this model, the intestinal barrier structure and functions indicators were maintained, thus providing a promising therapeutic potential.

Research perspectives

ANGPTL4 may be a valuable predictor, and similar research in patients who suffered critical care conditions could be evaluated.

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ORIGINAL ARTICLE

Retrospective Study

Prolonged survival in patients with hand-foot skin reaction secondary to cooperative sorafenib treatment

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Abstract

BACKGROUND

Sorafenib is an oral drug that prolongs overall survival (OS) in patients with hepatocellular carcinoma. Adverse events, including hand-foot skin reaction (HFSR), lead to permanent sorafenib discontinuation.

AIM

To clarify the association between interventions for adverse events and patient prognosis.

METHODS

We performed a retrospective, multicenter study of patients treated with sorafenib monotherapy between May 2009 and March 2018. We developed a mutual cooperation system that was initiated at the start of sorafenib treatment to effectively manage adverse events. The mutual cooperation system entailed patients receiving consultations during which pharmacists provided accurate information about sorafenib to alleviate the fear and anxiety related to adverse events. We stratified the patients into three groups: Group A, patients without HFSR but with pharmacist intervention; Group B, patients with HFSR and pharmacist interventions unreported to oncologists (nonmutual cooperation system); and Group C, patients with HFSR and pharmacist interventions known to oncologists (mutual cooperation system). OS and time to treatment failure (TTF) were evaluated using the Kaplan-Meier method.

RESULTS

We enrolled 134 patients (Group A, n = 41; Group B, n = 30; Group C, n = 63). The

Data sharing statement: The original anonymous dataset is available on request from the corresponding author at maochi-tei@umin.ac.jp.

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median OS was significantly different between Groups A and C (6.2 *vs* 13.9 mo, p < 0.01) but not between Groups A and B (6.2 *vs* 7.7 mo, $P = 0.62$). Group A *vs* Group C was an independent OS predictor (HR, 0.41; 95%CI: 0.25-0.66; $P < 0.01$). In Group B alone, TTF was significantly lower and the nonadherence rate was higher ($P < 0.01$). In addition, the Spearman's rank correlation coefficients between OS and TTF in each group were 0.41 (Group A; $P < 0.01$), 0.13 (Group B; $P = 0.51$), and 0.58 (Group C; $P < 0.01$). There was a highly significant correlation between OS and TTF in Group C. However, there was no correlation between OS and TTF in Group B.

CONCLUSION

The mutual cooperation system increased treatment duration and improved prognosis in patients with HFSR. Future prospective studies (*e.g.*, randomized controlled trials) and improved adherence could help prevent OS underestimation.

Key Words: Hepatocellular carcinoma; Sorafenib; Pharmacists; Oncologists; Prognosis; Duration of therapy

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Core Tip: We investigated the effect of cooperation between oncologists and pharmacists (mutual cooperation system) on the prognosis of patients with advanced hepatocellular carcinoma treated with sorafenib and found that cooperation increased medication adherence. Prolonged adherence was correlated with overall survival and time to treatment failure in patients with sorafenib-related hand-foot skin reactions. Our mutual cooperation system could be used to manage patients treated with various multikinase inhibitors and improve overall survival in studies that use sorafenib as the control drug. Future clinical investigations that include measures to improve medication adherence could eliminate the underestimation of medication efficacy that may otherwise occur due to preventable nonadherence.

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INTRODUCTION

Sorafenib is a multikinase inhibitor used to treat advanced hepatocellular carcinoma (HCC)[1,2]. Although sorafenib prolongs overall survival (OS) in patients with HCC, it is associated with various adverse events (AEs) that may lead to permanent discontinuation[3].

Previous studies found that hand-foot skin reaction (HFSR) was a prognostic marker of longer survival[4-6]. While HFSR is an important predictor of survival outcomes in clinical practice and clinical sorafenib trials, AE management could influence the efficacy of HFSR as a prognostic factor. A recent study showed that increased clinician experience with AEs reduced the potential for discontinuing sorafenib therapy, resulting in a longer OS in patients with HCC[7]. Nevertheless, it takes a long time for clinicians to develop the necessary experience for the management of AEs, and even with experience, it takes a substantial amount of time to provide a system of adequate follow-up after sorafenib initiation.

As sorafenib is administered orally, its successful use for HCC treatment relies on patient medication adherence. However, many studies indicate that patients with cancer are sometimes nonadherent when prescribed oral drugs[8,9], and AEs are the main cause of poor adherence[10]. Poor adherence can lead to poor outcomes, and clinicians may wrongly conclude that a drug is ineffective because the response to treatment is insufficient[11].

It is important for patients to actively participate in making treatment decisions and then receive treatment according to their decisions to improve adherence[12]. We introduced behavior change techniques (patient education, medication regimen management, pharmacist-led interventions)[13,14] in our facilities as interventions to promote adherence. Using a preliminary simulation, we estimated that collecting patient information takes at least 20 min. From this, we concluded that it is difficult for oncologists to manage drugs that cause various AEs (*e.g.*, sorafenib) without assistance due to their obligations to many patients. Thus, we developed a mutual cooperation system involving collaboration between oncologists and pharmacists to ensure effective AE management. This mutual cooperation system consisted of the initial intervention by a pharmacist followed by a medical examination by an oncologist. However, this system was affected by the patient's behavior because patients were not obliged to follow the system. Some patients received intervention from a pharmacist after a medical examination by an oncologist.

Effective AE management that improves medication adherence has a considerable impact on survival outcomes. Previous single-center studies suggest that healthcare provider interventions improve adherence, and the onset of HFSR was a favorable prognostic factor of OS in patients with HCC[15,16]. However, little is known about the association between prognosis and medication adherence in patients with HCC, and multicenter studies on this relationship are lacking. Therefore, we aimed to compare the impact of different AE interventions on patient prognosis.

MATERIALS AND METHODS

Study design

We retrospectively evaluated patients with advanced HCC treated with sorafenib monotherapy and no subsequent chemotherapeutic agent between May 2009 and March 2018 using the medical records of the following core hospitals in Japan: Hitachi General Hospital, Ibaraki Prefectural Central Hospital, Ibaraki Cancer Center, and Tokyo Medical University Ibaraki Medical Center. These hospitals are core hospitals that were designated by the government to provide specialized cancer medical care. The patients were separated into three groups: Group A, patients without HFSR but with pharmacist intervention (this intervention was performed by pharmacists who did not share interview information with the oncologist; it is called nonmutual cooperation system); Group B, patients with HFSR and nonmutual cooperation system; and Group C, patients with HFSR and intervention by pharmacists who shared interview information with the oncologist (mutual cooperation system).

Patient selection

We included patients with stage B or C HCC according to the Barcelona Clinic Liver Cancer (BCLC) staging system. The indication criteria for sorafenib administration were as follows: Child-Pugh grade A or B; Eastern Cooperative Oncology Group (ECOG) performance status 0 or 1; alanine aminotransferase < 5-fold the upper limit of the normal range; total bilirubin level < 2.0 mg/dL; neutrophil count > 1500/μL; hemoglobin level ≥ 8.5 g/dL; platelet count > 75000/μL; and no dialysis requirement. The study exclusion criteria were as follows: Patients with a history of thrombosis or ischemic heart disease, pregnant women and those who could become pregnant, and patients with brain metastases. Our study protocol was approved by the ethics committee of each hospital and was performed according to the ethical guidelines of the 1975 Declaration of Helsinki. We obtained informed consent using an opt-out option on each facility's website (see Institution website uniform resource locators). This study was registered with the University Hospital Medical Information Network (UMIN) (ID: UMIN000038701).

Data collection

We collected patient data from the start of sorafenib, including age, sex, etiology of underlying liver disease, Child-Pugh score, history of present illness, medical history, tumor marker level [alpha-fetoprotein (AFP)], ECOG performance status, and relevant laboratory tests, including total bilirubin, albumin, and international normalized ratio (INR). Laboratory tests and tumor marker levels were obtained every 8–10 wk until permanent sorafenib discontinuation.

Computed tomography evaluations

Sorafenib response evaluations on computed tomography (CT) were scheduled for 8 wk after the first treatment, and subsequent evaluations were planned every 8 wk. Thoracic, abdominal, and pelvic CT scans were performed with intravenous iodinated contrast media. CT evaluations were conducted based on the modified Response Evaluation Criteria in Solid Tumors (mRECIST)[17] by an oncologist.

Intervention

Pharmacists with special expertise provided medical care at the pharmacist's outpatient clinic before or after a patient was medically examined by an oncologist. Every 8 wk at each visit, an AE evaluation, a residual drug count, self-management advice, and patient education, including descriptions of successful cases of AE management, pharmacist support, and advice for relieving patient anxiety and misunderstanding, were conducted. AEs were evaluated according to the National Cancer Institute Common Toxicity Criteria for Adverse Events (NCI-CTCAE) version 5.0. Pharmacists recommended that all patients use heparinoids before sorafenib treatment to prevent AEs. Additionally, the prophylactic use of urea cream to prevent dermatologic AEs was recommended after beginning sorafenib treatment. Pharmacist intervention began at the start of sorafenib treatment and continued until treatment ended.

Mutual cooperation system

We developed a mutual cooperation system that was initiated at the start of sorafenib treatment to manage AEs effectively. Although patients in Groups B and C received medical advice from oncologists and pharmacists, the systems differed. Group C received 20- to 30-min consultations during which pharmacists provided accurate information about sorafenib to alleviate fear and anxiety related to AEs. After each visit, the pharmacist summarized the consultation results in a report and discussed their findings with an oncologist. Group B patients received a 5- to 10-min session during which pharmacists provided the same information about sorafenib that Group C had received. These pharmacist consultations were brief because a thorough medical examination by an oncologist had already been completed. Furthermore, the pharmacist did not record the consultation content in the medical chart because the visit involved verbal intervention only, and a detailed report was unnecessary.

Sorafenib therapy and AE management

Sorafenib was administered at a dose of 400 or 800 mg/d. The initial dose of sorafenib was determined by an oncologist. At the start of sorafenib treatment, all patients received information about AEs from a pharmacist and an oncologist. Patients who developed an AE could confer with their consulting pharmacist or prescribing oncologist. Pharmacists collected and recorded patient data, including the AE grade (according to NCI-CTCAE version 5.0), AE time of onset, and emotional response of the patient to the AE. Oncologists performed dose modifications throughout treatment, including reductions, interruptions, and reintroductions, according to the drug manufacturer's package insert for sorafenib.

Criteria for permanent sorafenib discontinuation

Sorafenib was permanently discontinued when any of the following events occurred: (1) Tumor progression, defined as either radiologic (by the mRECIST criteria) or clinically progressive disease (*e.g.*, ECOG performance status decline or onset of severe symptoms with no connection to liver failure); (2) Unacceptable AEs, defined as moderate to severe AEs (*e.g.*, grades 2–4) that persisted after dose reduction or temporary treatment interruption; or (3) Liver decompensation, defined as gastrointestinal bleeding, ascites, jaundice, or encephalopathy[3]. All patients were managed by an oncologist and received excellent supportive care after sorafenib was permanently discontinued. Time to treatment failure (TTF) was defined as the duration from the start of sorafenib treatment to permanent discontinuation. The proportion of days covered (PDC) was defined as the TTF divided by the time to radiologic progressive disease after sorafenib[18]. Nonadherence was defined as a PDC of ≤ 80%[19].

Statistical analysis

Categorical variables were assessed using the chi-square test and are presented as frequencies or percentages. Continuous variables were analyzed using the Mann-Whitney *U* test and are expressed as the mean ± SD. OS and TTF were evaluated using the Kaplan-Meier method. A landmark analysis[20] was performed to consider HFSR cases that might have occurred if a patient with HCC had not died as a guarantee-time

bias. The analysis was performed using the time when the highest-grade HFSR occurred in 50% or more of the patients as a landmark (here, 30 d). The log-rank test was used to estimate differences in survival curves. Additionally, we used Cox regression analyses to evaluate the relationship between the time to the occurrence of an event and explanatory variables. Logistic regression analyses were used to evaluate the relationship between nonadherence and explanatory variables.

We included the following baseline characteristics as variables in our univariate analysis: age, sex, etiology of liver disease, bilirubin level, albumin level, INR, BCLC stage, ECOG performance status, macrovascular invasion, extrahepatic spread, serum AFP level, and number of previous transarterial chemoembolization (TACE) procedures for liver cancer. Variables identified as significant in the univariate analysis were included in the multivariate analysis. The correlations between OS and TTF were assessed by Spearman's rank correlation coefficient. A p-value less than 0.05 was considered statistically significant. We used the Bonferroni correction for multiple comparisons to adjust the familywise error rate when comparing differences between the three groups. The statistical methods of this study were reviewed by Kamoshida T from the Department of Gastroenterology, Hitachi General Hospital, Japan. All statistical analyses were performed using SPSS software, version 22 (IBM Corp., Armonk, NY, United States).

RESULTS

Patients

We included 134 patients [median age, 69 years (range, 41–89 years); male, n = 99; female, n = 35] with advanced HCC who received sorafenib monotherapy without posttreatment (Group A, n = 41; Group B, n = 30; Group C, n = 63). The main etiological factor was hepatitis C virus (HCV) (77/134 patients, 57.5%), followed by hepatitis B virus (HBV) (30/134 patients, 22.4%).

Baseline characteristics

All patients had cirrhosis [Child-Pugh A, n = 117 (87.3%); Child-Pugh B, n = 17 (12.7%)]. HCC was BCLC stage B in 55 patients (41.0%) and BCLC stage C in 79 patients (59.0%). None of the patients had a second primary cancer. Portal vein thrombosis was present in 35 patients (26.1%), and extrahepatic metastases were found in 67 patients (50.0%) (Table 1).

AEs

An AE of at least grade 1 was observed in all patients after sorafenib administration. However, none of the patients experienced any grade 4 AEs. The main AEs in all groups were fatigue (30.6%), diarrhea (39.6%), hypertension (31.3%), anorexia (29.9%), and thrombocytopenia (38.8%) (Table 2). Many patients required temporary sorafenib interruption because of AEs (Group A, 19.5%; Group B, 6.7%; Group C, 41.3%). Of patients who temporarily stopped taking sorafenib, the rate of those who resumed treatment at a reduced dose was the highest in Group C (Group A *vs* Group B, P = 0.70; Group A *vs* Group C, P = 0.11; Group B *vs* Group C, P < 0.01) (Table 3).

Radiological response evaluations

CT examinations performed every 2 mo showed that the disease control rate (DCR) gradually decreased in all groups. The response rate (RR) and DCR 8 mo after the start of sorafenib treatment were the highest in Group C (RR, 9.5%; DCR, 65.1%) (Table 4).

Permanent sorafenib discontinuation

The main causes of permanent drug discontinuation were HCC progression and sorafenib-related AE intolerance. Permanent discontinuation due to AE intolerance occurred most frequently in Group B (Group A (17.1%) *vs* Group B (60.0%), P < 0.01; Group A (17.1%) *vs* Group C (20.6%), P = 1.00; Group B (60.0%) *vs* Group C (20.6%), P < 0.05) (Table 5).

OS after sorafenib therapy

The median OS was 6.2 mo in Group A, 7.7 mo in Group B, and 13.9 mo in Group C. The difference in the median OS was significant between Groups A and C (P < 0.01). In multivariate analysis, Group A *vs* Group C (HR, 0.41; 95%CI: 0.25–0.66; P < 0.01) and BCLC-B (HR, 0.60, 95%CI: 0.41–0.89; P = 0.01) were independent predictors of survival

Table 1 Baseline characteristics of patients in Groups A, B, and C

	Group A (*n* = 41)	Group B (*n* = 30)	Group C (*n* = 63)	*P* value
Age (yr)	70 (43–89)	67 (41–87)	69 (48–87)	0.233
Sex				
Male, *n* (%)	33 (80.5)	22 (73.3)	44 (69.8)	0.481
Child-Pugh class, *n* (%)				0.288
A	33 (80.5)	27 (90.0)	57 (90.5)	
B	8 (19.5)	3 (10.0)	6 (9.5)	
Etiology, *n* (%)				
HCV	22 (53.7)	11 (36.7)	44 (69.8)	0.235
HBV	9 (21.9)	10 (33.3)	11 (17.5)	0.417
Other	10 (24.4)	9 (30.0)	8 (12.7)	0.109
Portal vein thrombosis	14 (34.1)	10 (33.3)	11 (17.5)	0.099
Extrahepatic spread, *n* (%)	20 (48.8)	19 (63.3)	28 (44.4)	0.230
AFP (ng/mL), *n* (%)				
> 400	17 (41.5)	17 (56.7)	29 (46.0)	0.437
BCLC staging, *n* (%)				0.333
Stage B	15 (36.6)	10 (33.3)	30 (47.6)	
Stage C	26 (63.4)	20 (66.7%)	33 (52.4)	
ECOG performance status, *n* (%)				0.955
0	30 (75.6)	22 (73.3)	46 (73.0)	
1	10 (24.4)	8 (26.7)	17 (27.0)	
Total bilirubin (mg/dL)	0.99 ± 0.36	0.91 ± 0.37	0.83 ± 0.33	0.052
Albumin (g/L)	3.61 ± 0.50	3.73 ± 0.54	3.82 ± 0.49	0.301
INR	1.14 ± 0.11	1.15 ± 0.16	1.14 ± 0.19	0.481
Pre-sorafenib TACE procedures, *n* (%)				0.531
0	13 (31.7)	9 (30.0)	20 (31.8)	
1	3 (7.3)	6 (20.0)	13 (20.6)	
2	6 (14.6)	1 (3.3)	4 (6.4)	
3	4 (9.8)	2 (6.7)	8 (12.7)	
4	5 (12.2)	3 (10.0)	12 (19.0)	
> 5	10 (24.4)	9 (30.0)	6 (9.5)	

AFP: Alpha-fetoprotein; BCLC: Barcelona Clinic Liver Cancer; HCV: Hepatitis C virus; HBV: Hepatitis B virus; INR: International normalized ratio; TACE: Transcatheter arterial chemoembolization; ECOG: Eastern Cooperative Oncology Group.

(Figures 1 and 2, Table 6).

Mutual cooperation system evaluation

The median TTF in Group C was 5.0 mo (95%CI: 3.8–6.5), which was the highest of all the groups [Group C (5.0 mo) *vs* Group A (2.1 mo), $P < 0.01$; Group C (5.0 mo) *vs* Group B (0.5 mo), $P < 0.01$). In multivariable Cox regression analysis, Group A *vs* Group B (HR, 1.69, 95%CI, 1.04–2.75; $P = 0.03$) and Group A *vs* Group C (HR, 0.53; 95%CI: 0.35–0.81; $P < 0.01$) were significant predictors of TTF (Table 7). The proportions of patients with a PDC of < 0.8 were 29.3% in Group A, 73.3% in Group B, and 23.8% in Group C. Group B had a significantly higher sorafenib PDC than Groups A ($P < 0.01$) and C ($P < 0.01$). Adjusted logistic regression analysis showed that nonadherence (PDC ≤ 0.8) was associated with Group B *vs* Group A (OR, 0.11; 95%CI:

Table 2 Prevalence of adverse events after beginning sorafenib, according to CTCAE version 5.0, *n* (%)

	Group A (*n* = 41)				Group B (*n* = 30)				Group C (*n* = 63)			
	All grades	Grade 1	Grade 2	Grade 3	All grades	Grade 1	Grade 2	Grade 3	All grades	Grade 1	Grade 2	Grade 3
Any adverse event	37 (90.2)	30 (73.2)	18 (43.9)	5 (12.2)	30 (100)	28 (93.3)	24 (80.0)	14 (46.6)	63 (100)	58 (92.1)	36 (57.1)	14 (22.2)
Hand-foot skin reaction	0 (0)	0 (0)	0 (0)	0 (0)	30 (100)	15 (50.0)	10 (33.3)	5 (16.7)	63 (100)	39 (61.9)	19 (30.2)	5 (7.9)
Anemia	17 (41.5)	8 (19.5)	8 (19.5)	1 (2.4)	20 (66.6)	13 (43.3)	4 (13.3)	3 (10.0)	23 (36.5)	14 (22.2)	7 (11.1)	2 (3.2)
Diarrhea	15 (36.6)	12 (29.3)	3 (7.3)	0 (0)	11 (36.6)	9 (30.0)	1 (3.3)	1 (3.3)	27 (42.8)	21 (33.3)	2 (3.2)	4 (6.3)
Fatigue	14 (34.1)	10 (26.8)	3 (7.3)	0 (0)	11 (36.6)	7 (23.3)	4 (13.3)	0 (0)	16 (25.4)	10 (15.9)	6 (9.5)	0 (0)
Anorexia	14 (34.1)	10 (24.4)	3 (7.3)	1 (2.4)	7 (23.3)	5 (16.7)	1 (3.3)	1 (3.3)	19 (30.1)	15 (23.8)	4 (6.3)	0 (0)
Hypertension	12 (29.3)	10 (24.4)	2 (4.9)	0 (0)	7 (23.3)	2 (6.6)	5 (16.7)	0 (0)	23 (36.5)	16 (25.4)	4 (6.3)	3 (4.8)
Thrombocytopenia	9 (22.0)	2 (4.9)	4 (9.7)	3 (7.3)	23 (76.7)	10 (33.3)	8 (26.7)	5 (16.7)	20 (31.7)	10 (15.9)	5 (7.9)	5 (7.9)
Alopecia	6 (14.6)	4 (9.7)	2 (4.9)	0 (0)	2 (6.6)	2 (6.6)	0 (0)	0 (0)	20 (31.7)	19 (30.2)	1 (1.6)	0 (0)
Hepatic encephalopathy	1 (2.4)	0 (0)	1 (2.4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Table 3 Dose modification related to adverse events

	Group A (*n* = 41)	Group B (*n* = 30)	Group C (*n* = 63)	Group A *vs* Group B, *P* value	Group A *vs* Group C, *P* value	Group B *vs* Group C, *P* value
Dose reduction to initial dose of sorafenib	8/41 (19.5%)	2/30 (6.7%)	26/63 (41.3%)	0.700	0.108	0.005
Re-escalation to initial dose of sorafenib	2/8 (25.0%)	2/2 (100.0%)	2/26 (7.7%)	NA	NA	NA

NA: Not available.

Table 4 Radiological response according to the modified response evaluation criteria in solid tumors

	Group A (*n* = 41)	Group B (*n* = 30)	Group C (*n* = 63)
Complete response	0	1	1
Partial response	1	1	5
Stable disease	10	11	35
Progressive disease	30	17	22
Response rate	2.4%	6.7%	9.5%
Disease control rate	26.8%	43.3%	65.1%

0.04–0.36; $P < 0.01$) and Group B *vs* Group C (OR, 0.09; 95%CI: 0.03–0.27; $P < 0.01$) (Figure 3, Table 8).

Correlation between OS and TTF

The Spearman's rank correlation coefficients between OS and TTF in each group were 0.41 (Group A; $P < 0.01$), 0.13 (Group B; $P = 0.51$), and 0.58 (Group C; $P < 0.01$). There was a highly significant correlation between OS and TTF in Group C. However, there was no correlation between OS and TTF in Group B.

Table 5 Reasons for permanent sorafenib discontinuation, *n* (%)

	Group A (*n* = 41)	Group B (*n* = 30)	Group C (*n* = 63)	Group A *vs* Group B, *P* value	Group A *vs* Group C, *P* value	Group B *vs* Group C, *P* value
Progression	26 (63.4)	7 (23.3)	33 (52.4)	0.006	1.000	0.046
Intolerance	7 (17.1)	18 (60.0)	13 (20.6)	0.002	1.000	0.001
Liver failure	6 (14.6)	3 (10.0)	8 (12.7)	1.000	1.000	1.000
Other	2 (4.9)	2 (6.7)	9 (14.3)	1.000	0.690	1.000

Table 6 Prognostic factors of overall survival by multivariable Cox regression analysis

	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age (within 70 yr)	0.867	0.603–1.246	0.440	-	-	-
Male	1.216	0.802–1.842	0.357	-	-	-
Etiology (HBV)	1.313	0.809–2.133	0.271	-	-	
BCLC stage B	0.667	0.459–0.969	0.033	0.601	0.405–0.891	0.011
Portal vein thrombosis	1.677	1.092–2.575	0.018	1.133	0.674–1.903	0.638
Extrahepatic spread	0.740	0.509–1.074	0.113	0.671	0.419–1.076	0.098
AFP (> 400 ng/mL)	1.282	0.893–1.839	0.178	1.370	0.936–2.006	0.105
ECOG Performance status 1	0.752	0.488–1.158	0.196	1.042	0.636–1.708	0.869
Group A *vs* Group B	0.703	0.431–1.147	0.159	0.658	0.398–1.088	0.103
Group A *vs* Group C	0.431	0.281–0.663	< 0.001	0.407	0.253–0.654	< 0.001
Sorafenib administration period (second half *vs* first half)	1.205	0.840–1.728	0.311	-	-	-

AFP: Alpha-fetoprotein; BCLC: Barcelona Clinic Liver Cancer; HBV: Hepatitis B virus; ECOG: Eastern Cooperative Oncology Group.

DISCUSSION

We investigated the effect of cooperation between oncologists and pharmacists on the prognosis of patients with advanced HCC treated with sorafenib monotherapy. In the present study, the occurrence of HFSR was associated with improved patient prognosis, and this improvement was significantly enhanced by appropriate medication adherence. Close cooperation between oncologists and pharmacists increased adherence, and a strong correlation was observed between OS and TTF.

Several studies have indicated that the emergence of HFSR is associated with prolonged survival in patients with advanced HCC treated with sorafenib[5,6,21]. However, these studies did not evaluate the correlation between medication adherence and survival after the appearance of an AE, including HFSR. Targeted therapies, including sorafenib, can result in unexpected AEs that do not occur after the administration of earlier chemotherapy drugs[22]. Oncologists must recognize these novel AEs at an early stage and provide appropriate treatment to the extent possible. However, previous studies revealed that optimal AE management requires considerable experience and time[7,23]. Management of sorafenib-related AEs includes data collection for AE grading, patient education, and determination of the appropriate sorafenib dose by an oncologist[15].

The use of sorafenib is associated with various AEs, including gastrointestinal, constitutional, or dermatologic events[1,2], and their management may require dose reduction or temporary discontinuation to avoid sorafenib treatment cessation. For example, an appropriate sorafenib dose reduction yielded a decreased rate of permanent discontinuation due to AEs[7]. However, in many patients, these dose changes do not mitigate intolerable or severe AEs, and permanent sorafenib discontinuation is required[24].

Table 7 Prognostic factors of time-to-treatment failure by multivariable Cox regression analysis

	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age (within 70 yr)	1.059	0.747–1.501	0.747	-	-	-
Male	0.926	0.625–1.372	0.703	-	-	-
Etiology (HBV)	1.208	0.768–1.901	0.413	-	-	-
BCLC stage C	1.311	0.921–1.866	0.132	-	-	-
Portal vein thrombosis	1.379	0.925–2.056	0.115	1.011	0.662–1.545	0.958
Diarrhea	0.675	0.473–0.965	0.031	0.654	0.449–0.952	0.027
Hypertension	1.070	0.735–1.556	0.725	-	-	-
ECOG Performance status 1	0.687	0.446–1.058	0.089	0.725	0.463–1.135	0.159
Group B *vs* Group A	1.670	1.034–2.698	0.036	1.694	1.044–2.748	0.033
Group C *vs* Group A	0.495	0.328–0.747	< 0.001	0.529	0.346–0.811	0.003
Sorafenib administration period (second half *vs* first half)	0.980	0.694–1.384	0.908	-	-	-

AFP: Alpha-fetoprotein; BCLC: Barcelona Clinic Liver Cancer; HBV: Hepatitis B virus; ECOG: Eastern Cooperative Oncology Group.

Table 8 Prognostic factors of proportion of days covered by logistic regression analyses

	Adjusted analyses		
	OR	95%CI	*P* value
Male	0.352	0.141–0.877	0.025
Child-Pugh stage B	3.830	1.180–12.400	0.025
Diarrhea	0.472	0.198–1.120	0.089
Group B *vs* Group A	0.113	0.036–0.356	< 0.001
Group B *vs* Group C	0.091	0.031–0.266	< 0.001

In our study, only the mutual cooperation system promoted dose reduction after an AE and extended the TTF. In the mutual cooperation system, pharmacists were responsible for collecting data on AE grades, educating patients, and managing any leftover medicine. Furthermore, they documented their findings in a report for the oncologist. On the other hand, in the nonmutual cooperation system, an oncologist examined the patient before pharmacists were involved in patient management. Oncologists were required to evaluate AE grading data, educate patients, and determine the appropriate sorafenib dose. Only after the medical examination did a pharmacist provide additional patient management, and their findings were not reported to the oncologist. In the nonmutual cooperation system, the oncologist had to obtain a substantial amount of information to maintain or revise the sorafenib treatment regimen within 5 to 10 min.

Given these differences between the systems, our results suggest that the mutual cooperation system led to appropriate dose reductions, as reflected by the extended TTF. However, we do not recommend starting sorafenib at half the standard dose (800 mg/d). Dose reductions were guided by the results obtained by the mutual cooperation system. Additionally, we demonstrated that the best outcomes occur when optimal dosing and good medication adherence are achieved early in the course of sorafenib treatment. In our study, only the mutual cooperation system ensured good adherence in patients who experienced HFSR secondary to sorafenib treatment and prevented unnecessary permanent medication discontinuation.

A previous study showed that despite dramatic improvements in adjuvant hormonal therapy for breast cancer, nonadherence, early discontinuation, and effective cancer treatment are affected by treatment-related toxicity, and appropriate interventions are needed to improve breast cancer survival[25]. Another cohort study indicated that long-term tamoxifen therapy for breast cancer reduced the risk of death,

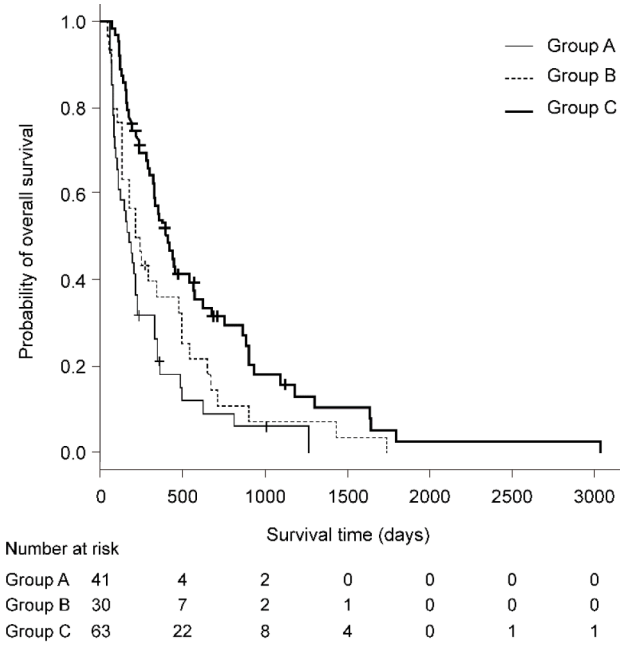


Figure 1 Kaplan-Meier estimates and prognostic factors of overall survival (comparison between each group). Group A, patients without hand-foot skin reaction (HFSR) but with pharmacist intervention; Group B, patients with HFSR and the nonmutual cooperation system; Group C, patients with HFSR and intervention by pharmacists who shared interview information with the oncologist (mutual cooperation system).

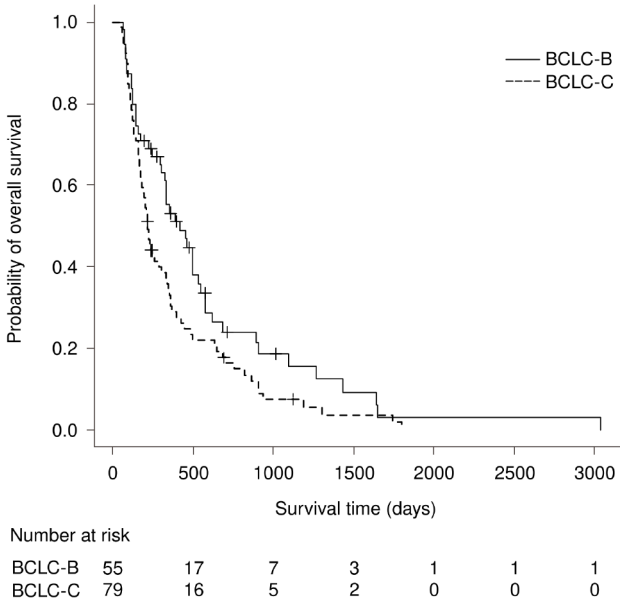


Figure 2 Kaplan-Meier estimates and prognostic factors of overall survival (Barcelona Clinic Liver Cancer B *vs* Barcelona Clinic Liver Cancer C). BCLC: Barcelona Clinic Liver Cancer.

while the risk of death increased with a low adherence rate[26]. In our study, the medical team continued sharing patient information after the patient started taking sorafenib; therefore, the mutual cooperation system enabled the medical team to prevent HFSR or promptly provide patient management, as appropriate. In contrast, the nonmutual cooperation system did not allow patient information to be shared at an early stage; thus, the medical team was not able to take measures to prevent HFSR or plan palliation care in a timely manner. The differences in the effectiveness of HFSR prevention and palliation between the two systems highlight the importance of the various hurdles that can affect medication adherence.

Hurdles to medication adherence are complex and include patient-, clinician-, and healthcare system-related factors. Patient-related factors, such as limited involvement in the treatment decision-making process, poor health literacy, doubts about

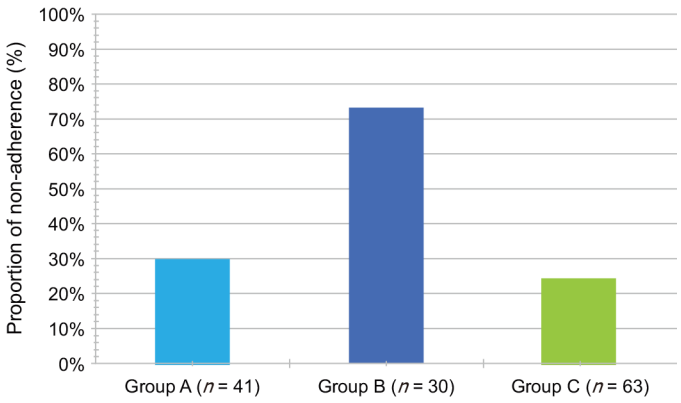


Figure 3 Proportion and prognostic factors of nonadherence. Group A, patients without hand-foot skin reaction (HFSR) but with pharmacist intervention; Group B, patients with HFSR and the nonmutual cooperation system; Group C, patients with HFSR and intervention by pharmacists who shared interview information with the oncologist (mutual cooperation system).

medication effectiveness, and previous adverse effects, influence adherence. Clinician-related factors include failure to recognize nonadherence, poor patient communication, and inadequate multidisciplinary communication between oncologists and pharmacists. Healthcare system-related factors include relationships with clinicians and clinicians' satisfaction with patient care[27,28]. Thus, multiple factors may become hurdles to improving adherence. The mutual cooperation system coordinates interactions among patients, clinicians, and the health system, thereby minimizing barriers to adherence.

Surprisingly, this study revealed that the prognostic value of HFSR was enhanced by appropriate medication adherence. On the other hand, BCLC-B HCC was an independent predictor of improved OS[29]. BCLC stage did not affect the difference in OS between Groups A and C, as there was no significant between-group difference in the baseline stage distribution.

We have reasonable evidence to confirm the validity of our results. First, variables such as age, sex, etiology, ECOG performance status, liver function, comorbidities, and TACE procedure count were not significantly different between the groups. Second, we verified that all patients had received sorafenib monotherapy and no subsequent chemotherapy; therefore, neither our patients' prognoses nor the prolonged OS we observed was affected by other chemotherapeutic agents.

Nevertheless, our study has a few limitations. First, our study design was based on the mutual cooperation system. After a patient was first checked by a specialized pharmacist, the oncologist determined whether to prescribe sorafenib based on the pharmacist's report. However, patients were not required to participate in the mutual cooperation system, and involvement was subject to the patient's wish. After the patients underwent a medical examination by an oncologist, a specialized pharmacist could also provide patient guidance about sorafenib. While patients who were unwilling to participate in the mutual cooperation system may have been included in Group A or B, it is unknown whether this enrollment could have affected the adherence rate. Second, OS and TTF were higher in the mutual cooperation system group (Group C) than in Group A and Group B. It is difficult to determine whether these results were caused by improved adherence or the mechanism underlying the prognostic efficacy of HFSR.

CONCLUSION

The mutual cooperation system increased treatment duration and improved prognosis in patients with HFSR secondary to sorafenib treatment. Additionally, the mutual cooperation system allowed us to promptly initiate sorafenib treatment. Our study clearly demonstrates the clinical and research benefits of this system. The mutual cooperation system for sorafenib treatment management described in this study could be applied to the management of patients treated with other multikinase inhibitors to extend OS. The increased OS resulting from the mutual cooperation system could have a substantial impact on the design of clinical studies in which sorafenib is used as the control drug. Additionally, nonadherence may have adversely affected OS in previous studies, leading researchers to underestimate drug efficacy. We propose that future

clinical investigations designed to improve medication adherence could eliminate OS underestimation.

ARTICLE HIGHLIGHTS

Research background

Although sorafenib prolongs overall survival (OS) in patients with hepatocellular carcinoma (HCC), the drug is associated with various adverse events (AEs) that may lead to permanent discontinuation.

Research motivation

The authors postulated that mutual cooperative intervention for AEs could improve OS in patients with HCC.

Research objectives

The aim of this study is to clarify the association between AE interventions and patient prognosis.

Research methods

The authors developed a mutual cooperation system that was initiated at the start of sorafenib treatment to manage AEs effectively. The system entailed pharmacist consultations during which patients were provided accurate information about sorafenib to alleviate fear and anxiety related to AEs. We stratified patients into three groups: Group A, patients without hand-foot skin reaction (HFSR) but with pharmacist intervention; Group B, patients with HFSR and pharmacist interventions unreported to oncologists (nonmutual cooperation system); and Group C, patients with HFSR and pharmacist interventions known to oncologists (mutual cooperation system).

Research results

The authors enrolled 134 patients (Group A, n = 41; Group B, n = 30; Group C, n = 63). The median OS significantly differed between Groups A and C (6.2 *vs* 13.9 mo, P < 0.01) but not between Groups A and B (6.2 *vs* 7.7 mo, P = 0.62). Group A *vs* Group C was an independent OS predictor (HR, 0.41; 95%CI: 0.25–0.66; P < 0.01). In Group B alone, the time to treatment failure (TTF) was significantly shorter, while the nonadherence rate was higher (P < 0.01). Additionally, Spearman's rank correlation coefficients between OS and TTF in each group were 0.41 (Group A; P < 0.01), 0.13 (Group B; P = 0.51), and 0.58 (Group C; P < 0.01). There was a highly significant correlation between OS and TTF in Group C. However, there was no correlation between OS and TTF in Group B.

Research conclusions

The mutual cooperation system increased the treatment duration and improved the prognosis of patients with HFSR.

Research perspectives

Future prospective studies (*e.g.*, randomized controlled trials) and improved adherence could help avoid OS underestimation.

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ORIGINAL ARTICLE

Retrospective Study

Contrast-enhanced ultrasound imaging for intestinal lymphoma

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Abstract

BACKGROUND

Intestinal lymphoma is a rare tumor. Contrast-enhanced ultrasound (CEUS) findings of intestinal lymphoma have not been reported previously, and the relationship between CEUS and clinicopathological features and prognostic factors is still unknown.

AIM

To describe the B-mode US and CEUS features of intestinal lymphoma and investigate the correlation of CEUS and histopathological features.

METHODS

This was a single-center retrospective study. Eighteen patients with histologically confirmed intestinal lymphoma underwent B-mode US and CEUS examinations between October 2016 and November 2019. We summarized the features of B-mode US and CUES imaging of intestinal lymphoma and compared the frequency of tumor necrosis in intestinal lymphomas with reference to different pathological subtypes (aggressive or indolent) and clinical stage (early or advanced). The time–intensity curve parameters of CEUS were also compared between patients with normal and elevated serum lactate dehydrogenase.

RESULTS

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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 do the examination by written consent.

Conflict-of-interest statement: There are no conflicts of interest to declare.

Data sharing statement: No additional data are available.

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In B-mode imaging, four patterns were observed in intestinal lymphoma: Mass type (12/18, 66.7%), infiltration type (1/18, 5.6%), mesentery type (4/18, 22.2%) and mixed type (1/18, 5.6%). All cases were hypoechoic and no cystic areas were detected. On CEUS, most cases (17/18, 94.4%) showed arterial hyperechoic enhancement. All cases showed arterial enhancement followed by venous wash out. A relatively high rate of tumor necrosis (11/18, 61.1%) was observed in this study. Tumor necrosis on CEUS was more frequent in aggressive subtypes (10/13, 76.9%) than in indolent subtypes (1/5, 20.0%) (P = 0.047). There were no correlations between tumor necrosis and lesion size and Ann Arbor stage. There was no significant difference in time–intensity curve parameters between normal and elevated lactate dehydrogenase groups.

CONCLUSION

B-mode US and CEUS findings of intestinal lymphoma are characteristic. We observed a high rate of tumor necrosis, which appeared more frequently in aggressive pathological subtypes of intestinal lymphoma.

Key Words: Intestinal lymphoma; Contrast enhanced ultrasound; B-mode ultrasound; Histopathological features; Lactate dehydrogenase; Quantitative diagnosis

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Core Tip: This is a small pilot study that described the appearance and pattern of intestinal lymphoma on B-mode ultrasound (US) and contrast-enhanced US. We revealed an unexpected contrast-enhanced US enhancement pattern with a high rate of tumor necrosis, which was found more frequently in aggressive than in indolent subtypes. This might provide additional information for clinical diagnosis and treatment of intestinal lymphoma.

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INTRODUCTION

Gastrointestinal tract is the most common site for extranodal lymphomas[1,2]. Approximately 20%–25% of primary extranodal lymphomas occur in the gastrointestinal tract [3,4]. Around 50%–70% of the gastrointestinal lymphomas arise in the stomach, 20%–30% in the small bowel and 5%–20% in the large bowel[5]. Primary intestinal lymphomas are less common than gastric lymphomas and differ in clinical features, pathology, treatment and prognosis[6-10]. There are few studies on the ultrasound (US) diagnosis of intestinal lymphoma, most of which are case reports. Intestinal tumors are usually studied through endoscopic or radiological imaging techniques, which may explain the lack of studies on US in intestinal lymphomas[11]. However, previous studies have already shown that US is an effective method for detecting gastrointestinal lesions, especially in experienced hands[12]. US-guided biopsy can also establish a definite pathological diagnosis, avoiding unnecessary surgery.

Contrast-enhanced US (CEUS) has emerged as a new imaging technique that can evaluate tumor vascularity[13]. CEUS is now widely accepted and recommended in inflammatory bowel disease in the European Federation of Societies for Ultrasound in Medicine and Biology (EFSUMB) guidelines. CEUS is suggested for assessment of the vascularity of the gastrointestinal wall and gastrointestinal tumors[14]. To the best of our knowledge, CEUS features of intestinal lymphoma have not previously been described. The aim of this study was to illustrate CEUS patterns of intestinal lymphoma for the first time and to investigate the relationship between CEUS and clinicopathological features and prognostic factors.

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MATERIALS AND METHODS

Patients

This is a single-center retrospective study based on a search of our imaging system from October 2016 to November 2019. Patients were excluded if they had a history of any other cancer or if they had previously received treatment. Written informed consent was obtained from each patient before the CEUS examinations.

Eighteen patients with histologically confirmed intestinal lymphoma were included, 10 men and 8 women, with age range 34–85 years. Definitive diagnosis for all the lesions was obtained by US-guided biopsy in 15 (83.3%) cases or by surgical resection in 3 (16.7%) cases. The diagnosis of intestinal lymphoma was based on the World Health Organization classification[15]. According to the Ann Arbor staging system, patients were classified into early stage (I/II) and advanced stage (III/IV). Pathological types were classified into aggressive and indolent lymphoma based on the World Health Organization classification. Primary intestinal lymphoma was defined as predominant intestinal lesion without previous peripheral lymphadenopathy or any lymphoma whose clinical presentation was related to intestinal involvement[16].

US examinations

All patients underwent B-mode US and CEUS with a 3.0–5.0 -MHz C5-1 probe for the iU22 sonography system (Philips Healthcare Ultrasound, Bothell, WA, United States) prior to surgery or biopsy. All patients were fasted for 8–12 h. CEUS was performed on all tumors under same protocols with the following parameters: Mechanical index 0.08–0.11, contrast gain 50%–60% and dynamic range 40 dB and focal zone was set at maximal depth. SonoVue (4.8 mL; Bracco, Milan, Italy) was administrated through a forearm vein in bolus within 1–2 s, followed by flush of 5 mL 0.9% saline. Images were acquired immediately after contrast agent was injected and lasted for 3 min and were recorded and stored as raw data on personal hard disks.

Image analysis

US and CEUS images were reviewed by two experienced radiologists, who were blinded to all clinical and pathological information. If there was disagreement between them, the final determination was made after discussion. The tumor size, internal echogenicity and presence of bowel wall thickening were recorded. Bowel wall thickening was defined as > 3 mm for small bowel and > 5 mm for large bowel. The enhancement pattern, enhancement distribution, tumor necrosis and presence of irregular and enlarged vessels (diameter > 5 mm) were recorded. The enhancement pattern was classified as homogeneous or heterogeneous. The enhancement distribution was clarified as diffuse, centripetal or centrifugal. Tumor necrosis was considered present if a nonenhanced area could be seen within the tumor during the arterial and venous phases.

According to the EFSUMB guidelines[14], the arterial phase is defined as the first 30 s after the contrast agent is injected, and the venous phase as the period between 30 and 120 s. By using Q lab software version 5 (Philips Medical Systems) on the workstation, we selected a region of interest (ROI) in the most enhanced area of the lesion, and the ROI area was set to 25 mm^2. The time–intensity curve (TIC) was automatically created for each ROI and then TIC parameters including rise time, time to peak, mean transit time, time from peak to one half, peak intensity, wash in slope and area under the curve were automatically obtained.

Statistical analysis

The Fisher's exact test was used to compare rate of tumor necrosis on CEUS between patients with Ann Arbor stage I/II and III/IV intestinal lymphomas and between patients with indolent and aggressive subtypes of intestinal lymphoma. Student's *t* test was used to compare the TIC parameters between normal and elevated serum lactate dehydrogenase (LDH) groups. Statistical significance was determined at $P < 0.05$ (two sided). Statistical analyses were performed using SPSS version 13.0 (SPSS Chicago, IL, United States).

RESULTS

Clinical and histological findings

This patient group comprised 18 patients with non-Hodgkin's lymphoma (NHL) of

various subtypes. Seventeen intestinal lymphomas were of B-cell origin; among which 11 were diffuse large B-cell lymphoma (DLBCL), 3 were follicular lymphoma, 2 were mucosa-associated lymphoid tissue lymphoma and 1 was mantle cell lymphoma. Only one case was of T-cell origin (enteropathy-associated T-cell lymphoma). No instances of intestinal Hodgkin's lymphoma were encountered. Primary intestinal lymphoma was diagnosed in 16 cases, whereas secondary lymphoma infiltration of the bowel was observed in 2 cases. Eight cases were classified as stage II, 4 as stage III and 6 as stage IV. The clinical presentations included abdominal pain (n = 7), palpable abdominal mass (n = 5), gastrointestinal bleeding (n = 2), constipation (n = 2), weight loss (n = 1) and fever (n = 1).

B-mode US

Four sonographic patterns of intestinal lymphoma were observed on B-mode imaging. The mass type (n = 12) presented as circumferential, marked bowel wall thickening forming masses, with loss of stratification, and hyperechoic lumen inside the mass (Figure 1A and B)[17,18]. The size of the masses ranged from 7.1 cm to 11.9 cm (mean 9.9 cm). The infiltration type presented as segmental bowel wall thickening without apparent mass formation (n = 1), with intestinal wall thickness of 2.6 cm (Figure 2A and B). The mesenteric type (n = 4) was characterized by multiple exophytic mesenteric masses with or without involvement of adjacent intestinal wall (Figure 3A and B)[19]. The lesion sometimes encapsulated mesenteric vessels without narrowing of the vascular lumen. The mixed type (n = 1) showed a combination of two patterns. Nine (50%) patients presented with bulky lesions (> 10 cm). Dilatation of the bowel was observed in 3 cases. Intestinal lymphomas were located in the small intestine in 5 cases, mesentery in f5, rectum in 1, ascending colon in 1, and 6 cases could not be clearly localized by US. Regional lymphadenopathy was found in 12 patients (66.7%), which manifested as aggregated nodules or masses in the mesentery or retroperitoneum.

CEUS

Rapid arterial enhancement followed by gradual washout in the venous phase was observed in all 18 patients (Figures 1C, 2C, and 3C). The peak enhancement was reached in the arterial phase or early venous phase. Seventeen cases presented with hyperechoic arterial enhancement, and only 1 case revealed hypoechoic arterial enhancement. Enhancement during the arterial phase was homogeneous (n = 7) or inhomogeneous (n = 11). Fifteen cases presented with diffuse enhancement, whereas three presented with centripetal enhancement. The visualization of irregular vessels was observed in 8 cases (44.4%). Tumor necrosis on CEUS was identified in 11 (61.1%) patients (Figure 1C), in which 1 case revealed extensive necrosis. Detailed information about B-mode US and CEUS findings of the 18 cases are shown in Table 1.

The mean diameter of the lesions with and without necrosis was 8.36 cm and 9.58 cm, respectively (P = 0.6). We compared the frequency of tumor necrosis in intestinal lymphomas with reference to different pathological subtypes or clinical stages. Tumor necrosis on CEUS was detected in 20.0% (1/5) of indolent intestinal lymphomas and in 76.9% (10/13) of aggressive subtypes (P = 0.047). Tumor necrosis was detected in 80% (8/10) of patients in III/IV stage and in 37.5% (3/8) patients in I/II stage (P = 0.145) (Table 2). Quantitative results of TIC parameters are shown in Table 3. The serum LDH value ranged from 130 to 968 U/L, with 10 patients' values exceeding the upper limit of normal (248 U/L). There was no significant difference in TIC parameters between normal and elevated LDH groups (Table 4).

DISCUSSION

In this study, we reported 18 cases of intestinal lymphoma. The mean age of our patients was 58.94 ± 15.89 years with most cases between 50 and 70 years. There was a male predominance with a male to female ratio of 1.25: 1. Lymphomas were predominantly located in the small bowel, and the most common symptoms included palpable abdominal mass and abdominal pain, which is in agreement with the literature[20]. All the intestinal lymphomas were NHL. B-cell lymphomas were more frequent than T-cell lymphomas (17: 1), and the most common pathological subtype was DLBCL (11/18, 61.1%). These results correlated well with previous studies[3,21].

We confirmed some of the sonographic features reported earlier and provided new information regarding the CEUS findings in intestinal NHL. Our data showed that mass type was the most frequent form of intestinal NHL on B-mode imaging (12/18,

Table 1 B-mode ultrasound and contrast-enhanced ultrasound patterns and clinicopathological characteristics of intestinal lymphoma in 18 patients

No.	Age (yr)	Sex	Location	Pathological subtype	B-mode US pattern	CEUS enhancement pattern	Necrosis
1	78	M	Ileocecal region	DLBCL	Mass type	Diffuse, inhomogeneous, arterial hyperechoic uptake. gradually venous wash-out	Yes
2	34	M	Rectum	MATL	Mass type	Diffuse, inhomogeneous, arterial hyperechoic uptake. gradually venous wash-out	Yes
3	43	M	Mesentery	DLBCL	Mesenteric type	Diffuse, inhomogeneous, hyperechoic arterial uptake. gradually venous wash-out	Yes
4	46	M	Small intestine	FL	Mass type	Diffuse, homogeneous, hypoechoic, arterial uptake. gradually venous wash-out	No
5	75	F	Ascending colon	DLBCL	Infiltration type	Diffuse, homogeneous, hyperechoic arterial uptake. gradually venous wash-out	No
6	78	F	Jejunum, ileum	EATL	Mass type	Diffuse, inhomogeneous, hyperechoic arterial uptake. gradually venous wash-out	Yes
7	52	F	Jejunum	DLBCL	Mass type	Diffuse, inhomogeneous, hyperechoic arterial uptake. gradually venous wash-out	Yes
8	74	M	Small intestine	DLBCL	Mass type	Diffuse, inhomogeneous, hyperechoic arterial uptake. gradually venous wash-out	Yes
9	52	M	Jejunum, ileum	MATL	Mass type	Diffuse, homogeneous, hyperechoic arterial uptake. gradually venous wash-out	No
10	61	M	Mesentery	DLBCL	Mesenteric type	Diffuse, inhomogeneous, hyperechoic arterial uptake. gradually venous wash-out	Yes
11	41	F	Mesentery	DLBCL	Mesenteric type	Diffuse, inhomogeneous, hyperechoic arterial uptake. gradually venous wash-out	No
12	46	F	Small intestine	DLBCL	Mass type	Diffuse, inhomogeneous, hyperechoic arterial uptake. gradually venous wash-out	Yes
13	64	M	Ileocecum, ascending colon	DLBCL	Mass type	Diffuse, inhomogeneous, hyperechoic arterial uptake. gradually venous wash-out	Yes
14	68	F	Small intestine	DLBCL	Mixed type	Diffuse, inhomogeneous, hyperechoic arterial uptake. gradually venous wash-out	Yes
15	61	F	Small intestine	FL	Mass type	Diffuse, homogeneous, hyperechoic arterial uptake. gradually venous wash-out	No
16	85	M	Small intestine	MCL	Mass type	Diffuse, homogeneous, hyperechoic arterial uptake. gradually venous wash-out	No
17	35	M	Mesentery	FL	Mesenteric type	Diffuse, homogeneous, hyperechoic arterial uptake. gradually venous wash-out	No
18	68	F	Small intestine	DLBCL	Mass type	Diffuse, homogeneous, hyperechoic arterial uptake. gradually venous wash-out	Yes

CEUS: Contrast-enhanced ultrasound; DLBCL: Diffuse large B-cell lymphoma; FL: Follicular lymphoma; MCL: Medial collateral ligament; MATL: Melanoma-associated T-lymphocytes; EATL: Enteropathy associated T-cell lymphoma.

66.7%), which was in agreement with previous studies[22]. However, this finding was not specific because various intestinal abnormalities also can lead to bowel wall thickening. Nevertheless, bowel wall thickening in lymphoma is often significant (> 2 cm) compared to other malignancies and often located in the ileocecal region, with coexistence of perivisceral multiple lymph nodes. Diffuse or segmental bowel wall thickening is characteristic of intestinal lymphoma, especially when associated with bowel lumen dilation, but this US pattern only comprised 5.6% in our study.

In our study, rapid arterial enhancement followed by elimination of the contrast media in the venous phase was observed in all cases, which is similar with other malignancies. This finding is believed to be correlated with the presence of arteriovenous shunts and a well-represented circulatory bed. Thirteen cases exhibited diffuse enhancement, in accordance with previous studies[23]. Most (17/18, 94.4%) intestinal lymphomas displayed hyperechoic arterial enhancement, which is different from isoechoic or hypoechoic arterial enhancement observed in lymphoma infiltration

Table 2 Tumor necrosis and tumor size, clinical stage and pathological subtypes

Characteristic	With necrosis	Without necrosis
Tumor size (cm)	8.36	9.58
Stage		
Early stage	3	5
Advanced stage	8	2
Subtype[a]		
Indolent subtype	1	4
Aggressive subtype	10	3

[a]$P = 0.047$.

Table 3 Time–intensity curve parameters of contrast-enhanced ultrasound

TIC parameters	Range	mean ± SD
Rise time (s)	2.90-27.62	13.74 ± 6.11
Time to peak (s)	7.64-36.00	24.41 ± 6.61
Mean transit time (s)	29.94-77.75	49.94 ± 13.98
Time from peak to one half (s)	36.38-127.85	77.88 ± 24.12
Peak intensity (dB)	7.8-29.28	17.57 ± 5.98
WIS (dB/s)	0.53-29.28	1.35 ± 1.09
AUC	590.99-3616.03	1772.05 ± 824.89

AUC: Area under curve; WIS: Wash in slope; TIC: Time–intensity curve.

Table 4 Time–intensity curve parameters and serum lactate dehydrogenase level

TIC parameters	Normal LDH	Elevated LDH	*P* value
Rise time (s)	14.34	12.78	0.693
Time to peak (s)	27.17	27.07	0.982
Mean transit time (s)	53.39	46.21	0.382
Time from peak to one half (s)	82.70	72.49	0.442
Peak intensity (dB)	16.50	16.68	0.955
Wash in slope (dB/s)	1.38	1.26	0.824
Area under the curve (dBs)	1699.47	1712.42	0.979

TIC: Time–intensity curve; LDH: Lactate dehydrogenase.

of the spleen, lungs and kidneys[24-26]. The reason for this discrepancy is unclear and needs to be confirmed in larger populations.

It is notable that there was a high rate of necrosis (11/18, 61.1%) on CEUS, which is inconsistent with previous studies. Despite necrosis being prone to occur in large tumors, we found no correlation between the presence of tumor necrosis on CEUS and tumor size. Necrosis was not detected in 4 of the 9 cases with bulky lesions. Necrosis may indicate aggressive tumor growth, and thus be associated with poor prognosis. Some researchers have explored the prognostic implications of tumor necrosis at computed tomography (CT) or positron emission tomography in lymphoma. Saito *et al* [27] did not find an association between disease-free survival and presence of necrosis in lymph nodes in 60 patients with different NHL subtypes[27]. However, Adams *et al*

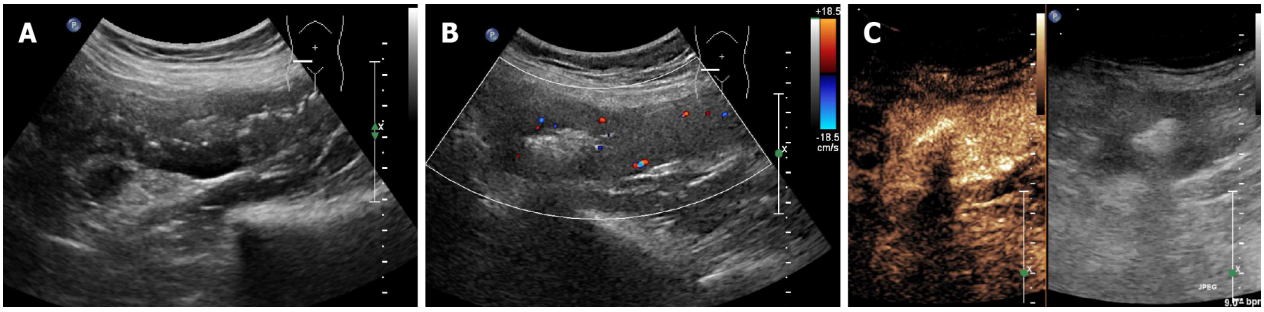


Figure 1 A 78-year-old female patient (case No. 6) with primary enteropathy-associated T-cell lymphoma in small intestine. A: B-mode imaging shows polypoidal lesions presenting "pseudo renal sign"; B: Color Doppler flow imaging shows abundant blood flow in bowel wall; C: Contrast-enhanced ultrasound showing inhomogeneous intense arterial enhancement with small non enhanced area.

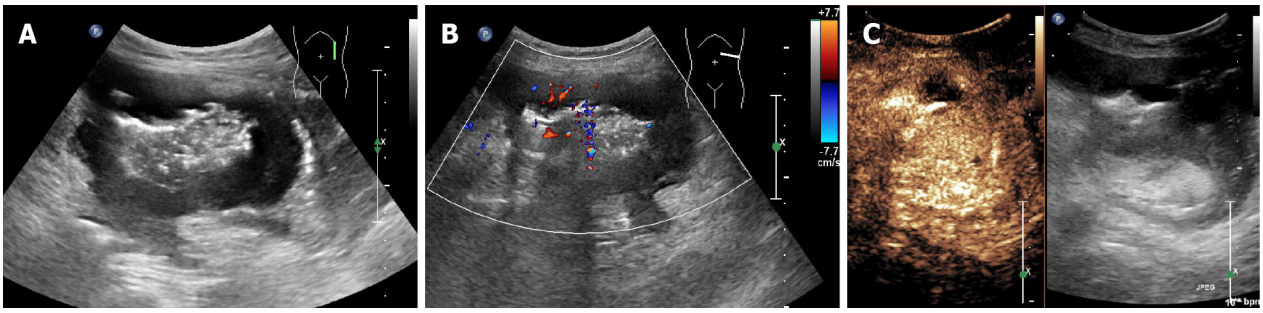


Figure 2 A 75-year-old female patient (case No. 5) with primary diffuse large B-cell lymphoma in the terminal ileum and ascending colon. A: B-mode imaging show circumferential bowel wall thickening with dilation of the bowel; B: Color Doppler flow imaging shows abundant blood flow in bowel wall; C: Contrast-enhanced ultrasound showing homogeneous intense arterial enhancement.

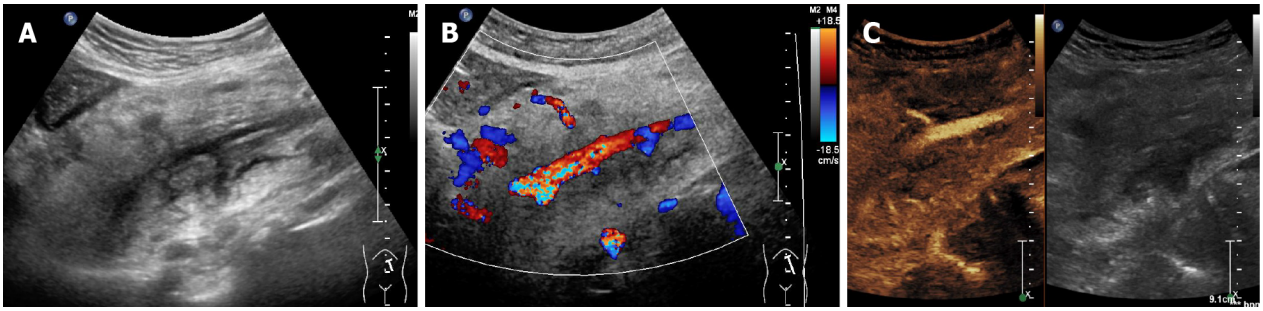


Figure 3 A 68-year-old female patient (case No. 14) with secondary diffuse large B-cell lymphoma intestinal lymphoma in mesentery. A: B-mode imaging shows large mesenteric mass encapsulating mesenteric vessels with ill-defined echoic region; B: Color Doppler flow imaging shows the abdominal aorta and its branches; C: Contrast-enhanced ultrasound showing inhomogeneous moderate arterial enhancement.

[28] showed that patients with newly diagnosed DLBCL with tumor necrosis at CT had significantly worse progression-free survival and overall survival than patients without tumor necrosis at CT. The National Comprehensive Cancer Network International Prognostic Index score did not differ significantly between the two groups. Our study showed that the frequency of necrosis on CEUS was different between indolent and aggressive lymphoma subtypes, but it was not related to clinical stage. Since it as a retrospective study, patients were not followed up to assess prognosis.

Evaluation of prognosis by imaging is desirable but often not possible. It is known that tumor growth is accompanied by neoangiogenesis. The assessment of the bowel vasculature can provide functional information about tumor perfusion, particularly with the advent of the second generation of contrast agents for CEUS. The generation of TICs allows for objective measurement of blood flow parameters[29]. In our previous study, we demonstrated that enhanced intensity is associated with microvascular density in rectal cancer[30]. In the present study, we investigated the relationship between TIC parameters and serum LDH level. LDH can reflect tumor load and is an independent prognostic factor of lymphoma[31]. However, we found no significant

difference in TIC parameters between normal and elevated serum LDH groups.

US is not the method of choice for the diagnosis of intestinal lymphoma, because it may not detect small or deep lesions that can be obscured by bowel gas; it is often used as targeted US for evaluation of suspected region but has difficulty in thorough assessment and staging for advanced lymphoma. Intestinal lymphoma is conventionally diagnosed by CT, magnetic resonance imaging and positron emission tomography/CT. However, CEUS used with second-generation contrast agent, offered a reasonable alternative imaging solution for those who are not suitable for nephrotoxic iodine-based contrast medium injection. Moreover, in our experience, CEUS guided biopsy allows targeting viable regions of the tumor; it has significantly improved our ability to obtain sufficient tissue for immunohistochemistry and molecular testing, which is particularly important in diagnosis and evaluation of lymphoma.

This study had some limitations. Intestinal lymphoma is a rare entity and the sample size was small. Furthermore, the patients included in this study who underwent CEUS examinations were usually referred for US-guided biopsy, which may have led to selection bias. As it was a retrospective study, some clinical data were missing and prognostic information was not included.

CONCLUSION

In this study, our findings on intestinal lymphoma contribute to knowledge of CEUS imaging. In this small group, intestinal lymphomas showed a characteristic enhancement pattern with rapid wash followed by early wash out of the contrast media. A high rate of tumor necrosis was detected, with a tendency to be more likely in aggressive subtypes, which needs to be confirmed in a larger population. Although US is not the primary imaging modality in diagnosis of intestinal lymphoma, it can play an important role in guidance of biopsy and provide more information.

ARTICLE HIGHLIGHTS

Research background

Contrast-enhanced ultrasound (CEUS) is a new imaging technique that can evaluate tumor vascularity, and it has been proved to have value in the diagnosis and assessment of inflammatory bowel disease. However, the CEUS features of intestinal lymphoma remain unclear.

Research motivation

There have been few studies on CEUS findings of intestinal lymphoma and their potential correlation with histopathological features.

Research objectives

Our present study aimed to describe the B-mode US and CEUS features of intestinal lymphoma and investigate the correlation of CEUS and histopathological features.

Research methods

We summarized the features of B-mode US and CUES imaging of intestinal lymphoma of 18 patients and compared the frequency of tumor necrosis in intestinal lymphomas with reference to different pathological subtypes (aggressive or indolent) and clinical stage (early or advanced). Besides, the time–intensity curve parameters of CEUS were also compared between patients with normal and elevated serum lactate dehydrogenase (LDH).

Research results

In B-mode imaging, four patterns were observed in intestinal lymphoma: Mass type (12/18, 66.7%), infiltration type (1/18, 5.6%), mesentery type (4/18, 22.2%) and mixed type (1/18, 5.6%). All cases were hypoechoic and no cystic areas were detected. On CEUS, most cases (17/18, 94.4%) showed arterial hyperechoic enhancement. All cases showed arterial enhancement followed by venous wash out. A relatively high rate of tumor necrosis (11/18, 61.1%) was observed in this study. Tumor necrosis on CEUS was more frequent in aggressive subtypes (10/13, 76.9%) than in indolent subtypes (1/5, 20.0%) (P = 0.047). There were no correlations between tumor necrosis and lesion

size and Ann Arbor stage. There was no significant difference in time–intensity curve parameters between normal and elevated LDH groups.

Research conclusions

B-mode US and CEUS findings of intestinal lymphoma are characteristic. We observed a high rate of tumor necrosis, which appeared more frequently in aggressive pathological subtypes of intestinal lymphoma.

Research perspectives

Based on the present results, we will expand the number of enrolled patients and explore the advantages of US compared with other imaging techniques in diagnosing intestinal lymphoma.

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ORIGINAL ARTICLE

Observational Study

Intestinal ischemic manifestations of SARS-CoV-2: Results from the ABDOCOVID multicentre study

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Abstract

BACKGROUND

Intestinal ischemia has been described in case reports of patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) disease (coronavirus disease 19, COVID-19).

AIM

To define the clinical and histological, characteristics, as well as the outcome of ischemic gastrointestinal manifestations of SARS-CoV-2 infection.

METHODS

A structured retrospective collection was promoted among three tertiary referral centres during the first wave of the pandemic in northern Italy. Clinical, radiological, endoscopic and histological data of patients hospitalized for COVID-19 between March 1st and May 30th were reviewed. The diagnosis was established by consecutive analysis of all abdominal computed tomography (CT) scans performed.

RESULTS

Among 2929 patients, 21 (0.7%) showed gastrointestinal ischemic manifestations either as presenting symptom or during hospitalization. Abdominal CT showed bowel distention in 6 patients while signs of colitis/enteritis in 12. Three patients presented thrombosis of main abdominal veins. Endoscopy, when feasible, confirmed the diagnosis (6 patients). Surgical resection was necessary in 4/21 patients. Histological tissue examination showed distinctive features of endothelial inflammation in the small bowel and colon. Median hospital stay was 9 d with a mortality rate of 39%.

CONCLUSION

Gastrointestinal ischemia represents a rare manifestation of COVID-19. A high index of suspicion should lead to investigate this complication by CT scan, in the attempt to reduce its high mortality rate. Histology shows atypical feature of ischemia with important endotheliitis, probably linked to thrombotic microangiopathies.

Key Words: Coronavirus; COVID-19; Ischemic colitis; Small bowel ischemia; Endothelial inflammation

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Core Tip: Ischemic manifestations have been described as possible presenting symptoms of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. These manifestations are frequent in the respiratory tract and bear a high fatality rate. Our retrospective observational trial aims to describe the prevalence, the characteristics and the evolution of patients presenting with intestinal ischemic manifestations of SARS-CoV-2 infection.

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INTRODUCTION

The end of December 2019 marked the recent world history for the advent of a new zoonosis caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). SARS-CoV-2 rapidly spread around the world causing a pandemic responsible for a dramatic number of deaths due to a disease referred to as "coronavirus disease 19 (COVID-19)" (https://www.healthmap.org/covid-19/).

The Lombardy region (Italy) was the second epicentre of the pandemic[1]. A recent report of the Italian National Institute of Statistics revealed that in March 2020, there was an overall increase in the general population mortality rate of + 185% (+ 568% in the Bergamo province) compared to the same period of the previous 4 years (2015-2019) (https://www.istat.it/it/files//2020/05/Rapporto_Istat_ISS.pdf).

The most frequent clinical manifestation of COVID-19 is an atypical pneumonia, presenting with respiratory symptoms such as fever, cough, shortness of breath and hypoxia[2]. However, several cohort studies worldwide showed that in a minority of cases COVID-19 can present with isolated gastrointestinal symptoms (GI), including abdominal pain, nausea, vomiting and diarrhoea[3-6]. A metanalysis demonstrated a poorer prognosis of COVID-19 patients presenting with gastrointestinal symptoms, compared to other COVID-19 patterns of presentation[7]. This may be due to the severe intestinal ischemic lesions found in 3 of the first 12 autopsies performed in COVID-19 patients in Germany[8]. Furthermore, a preliminary report highlighted that, during the first wave of SARS-CoV-2 pandemic, there was a higher prevalence of gastrointestinal ischemic events compared with the same timeframe before the virus advent[9].

During the first wave of the pandemic the medical community was unaware of the multifaceted manifestations of this new infectious agent. Now that the world has faced the second wave of this pandemic, it is crucial to identify retrospectively all the possible life-threatening manifestations of SARS-CoV-2 for which appropriate management may influence the final outcome.

The aim of the present multicentre study, named ABDOCOVID, is to analyse retrospectively the main clinical, endoscopic, pathological and imaging findings in patients with intestinal ischemic manifestations of SARS-CoV-2, in three tertiary referral centres of the highly hit region of Lombardy.

MATERIALS AND METHODS

Patients and methods

All patients admitted from March 1st to May 30th to the emergency department (ED) of one of the three enrolling Hospitals (Papa Giovanni XXIII, Bergamo; Humanitas Clinical and Research Center, IRCCS, Rozzano, Milano; Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan) and with confirmed SARS-CoV-2 infection, were reviewed and analysed. All confirmed COVID-19 patients who underwent abdominal computed tomography (CT) scan or endoscopy were evaluated, and data on the diagnostic workup were retrospectively collected.

In order to calculate the prevalence of intestinal ischemic manifestations, aggregate data on all patients admitted for COVID-19 in the three enrolling Hospital were pooled.

The local Ethical Committee approved the study protocol (protocol 96/20). All patients' data were anonymized and treated in accordance with the declaration of Helsinki.

Clinical data collection

The medical notes of enrolled patients were examined to gather information on their past medical history, symptoms at presentation to the ED, concomitant pulmonary findings and their evolution. In addition, clotting parameters of the patients, such as prothrombin time, partial thromboplastin time, platelet count and D-dimer level were collected.

Imaging acquisition protocol and analysis

All abdominal CT scans performed at Papa Giovanni XXIII Hospital (Bergamo) were acquired with the patient in supine position, on a 64-detector scanner (Revolution EVO; GE Medical), in the ED. All CTs included at least a portal venous phase, acquired 80-90 s following the injection of a non-ionic contrast medium (iomeprol 350 mg/mL, Bracco Imaging, Italy), in an antecubital vein. The mean contrast dose was 1.3-1.5 mL per patient's kg, with a flow rate of 3/3.5 mL/s. A rapid saline solution flush (about 30/40 mL, flow rate at least 3 mL/s) was injected thereafter. In specific cases (suspected bowel ischemia or bleeding), an unenhanced scan and an arterial phase were additionally performed. The CT scanning protocol and contrast medium characteristics were similar for Humanitas (64-detector scanner, Philips Brilliance) and Policlinico (Flash 64 and SOMATON 256, Siemens) cohorts of patients.

Image analysis was performed by expert radiologists: Papa Giovanni XXIII: 2 board-certified radiologists in consensus (Bonaffini PA, and Valle C, 12 and 6 years of experience in abdominal imaging, respectively); Humanitas: 1 radiologist (Bonifacio C, 17 years of experience in abdominal imaging); Ospedale Maggiore Policlinico: 1 radiologist (Forzenigo L, 20 years of experience in abdominal imaging).

On CT scans of enrolled patients we evaluated the following parameters: (1) Small bowel distension, with corresponding maximum calibre (mm) and extension (ileal, jejunal, both); (2) Large bowel distension, with corresponding maximum calibre (mm) and extension (cecum, ascending, transverse, descending, sigmoid colon, rectum, all); (3) Thrombosis in the inferior vena cava (IVC), superior mesenteric vein (SMV) and portal vein (PV) and/or in the superior mesenteric artery (main trunk and branches); (4) Intraluminal air in SMV and/or PV; (5) Bowel wall characteristics (preserved/absent enhancement, layered enhancement, thickening, parietal pneumatosis); (6) Pneumoperitoneum and/or ascites; and (7) Other ischemic sites in the abdomen (either vessels or organs). At the same time, the concomitant presence of pulmonary embolism (PE) with binary score (yes/no) and lung disease stages at chest CT were also recorded, if present. Lung involvement was classified as early, progressive, peak or absorption, as previously reported by Pan *et al*[10].

Endoscopic screening and findings

Endoscopic procedures were performed only in patients with COVID-19 GI symptoms and concomitant clinical history or radiological findings compatible with suspected colitis. All procedures were performed by expert endoscopists for each centre (Indriolo A, Elli L, Furfaro F, experienced endoscopists for more than 10 years). All endoscopic procedures on COVID-19 positive patients were organized in a COVID-19 dedicated operating room or in intensive care unit, depending on patients' clinical conditions. Appropriate protection equipment was worn to perform the endoscopic procedures, and the ventilators used to assist the patients were sanitized according to internal antiseptic protocols.

At GI endoscopy we recorded data on the aspects of ischemic colitis such as haemorrhagic purple nodules, indicating submucosal bleeding. Other common endoscopic findings in cases of ischemic colitis could be petechiae, oedema, easily friable mucosa, and development of pseudo membranes[11-13].

Pathology findings

When available, histological specimens from intestinal biopsies, surgical resection, autoptic samples were analysed. All biopsies were reviewed by the same pathologist with over 20 years of experience in intestinal biopsy interpretation. In addition to routine staining and fixation, CD34 immunostaining for endothelial cells identification was performed systematically on all specimens.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 21. Categorical variables were compared using Chi Square. Correlations were analysed by Pearson or Spearman test depending on parametric or non-parametric variables. Proportions are reported with 95% exact binomial confidence intervals (CI). To compare mortality rate between COVID-19 patients with and without intestinal ischemia we used risk ratios as an effect measure with 95%CI calculated by the Fisher's exact test.

RESULTS

Clinical characteristics upon admission

During the study period, 2929 patients with COVID-19 were admitted to the three enrolling centres. GI presenting symptoms were found in 16% (95%CI: 0.14-0.17) of patients. We identified 21 patients (66% male, median age 69 years, range 62-79), who met the aforementioned inclusion criteria. The prevalence of intestinal ischemia in COVID-19 hospitalized patients was 0.7% (95%CI: 0.04-0.10). Baseline characteristics of the patients are presented in Table 1.

All but one patient presented with positive SARS-CoV-2 nasopharyngeal swab. SARS-CoV-2 diagnosis in one patient was made through SARS-CoV-2 RNA detection in the intestinal mucosa[14]. All patients were admitted to the Hospital after an ED access. In 11/21 (52%) cases, the clinical picture was characterized by gastrointestinal symptoms, being abdominal pain the most represented (5/11, 45%). Two patients did not present any comorbidity, while the most prevalent in the remainders was hypertension (in 10/21, 48%).

Eight of 21 patients (38%) were under anticoagulants or antiplatelets therapies at the time of ED admission. D-dimer was elevated in 17/21 (81%) of patients, while the rest of coagulation parameters were compatible with their treatment.

Radiological findings

Bowel distension was evident in 45% of cases. In 6/20 (30%) patients there was small bowel distension (mean maximum diameter 41.2 mm; range 30-56), generally involving the ileum (4/6 cases) and diffuse in 1 case. Large bowel was distended (mean diameter 94 mm; range 91-97) in 3/20 (15%) patients (2 with associated ileal involvement): In 2 cases the dilatation extended from transverse to cecum, while in 1 it involved the whole colon (Figure 1).

Six patients (30%) had CT scan evidence of small or large bowel ischemia, with reduced wall thickness and enhancement. In four of these cases there was parietal pneumatosis with associated loop over distension (3 small, 2 large bowel). One patient had pneumoperitoneum. In four cases (3 with bowel ischemia) there were associated splenic (3) or renal (1) parenchymal infarcts.

Twelve patients (60%) had CT evidence of colitis/enteritis, with layered enhancement (mucosal enhancing, submucosal oedema) and wall thickening, (mean thickness 10.9 mm; range 7-17). The most commonly involved sites were left/sigmoid colon and/or terminal ileum; one patient had pancolitis. One patient demonstrated the same features in the oesophagus.

In the majority of cases (13/20, 65%) there was no evidence of venous or arterial acute thrombosis, including almost all patients with CT scan signs of bowel ischemia or colitis/enteritis. Thrombosis of a main abdominal vein was present only in three patients: IVC with SMV (one case with SB ischemia), PV with SMV (1) and PV alone (1). Three patients had iliac-femoral veins thrombosis, without involvement of any other vessel. In one case, we observed an acute splenic artery thrombosis, with associated parenchymal infarcts. None of these patients had evidence of intraluminal arterial or venous air, hematomas or signs of bleeding in the abdomen. In eight cases ascites was present.

Five patients had simultaneous evidence of PE but in 15/21 cases the assessment of pulmonary artery was not feasible. Pulmonary stage evaluation from chest CT was feasible in 11/21 (52%) patients: The most common one was early (5/11), followed by progressive (3/11), peak (2/11) and absorption (1/11).

Endoscopic findings

Endoscopy was performed in 8/21 (38 %) patients. Five (63%) demonstrated typical features of ischemic colitis with active bleeding from an oedematous mucosa, with petechiae in three of them and mucosal oedema and ischemic ulceration in the remaining 2 (Figure 2). Neither patchy lesions nor stenotic complication of ischemia were found in any of the performed endoscopies.

Histopathology findings

In three colic biopsies collected from four patients' mucosal lamina propria oedema, blood congestion, coagulative epithelial necrosis and ulcerations, intramucosal haemorrhagic foci were observed and interpreted as suggestive of acute ischemic injury. One biopsy showed normal histology; however, endoscopy was performed 2 mo after admission. One specimen from small bowel resection and one from autopsy were analysed. Both showed extensive haemorrhagic necrosis of mucosal layer,

Table 1 Patients clinical characteristics upon admission

Patients	Age	Sex	Comorbidities	Ongoing ACT/APT	Entry symptoms	D-dimer
1	85	M	HT, CVD	Yes	Low GI bleeding	Not done
2	71	F	HT	Yes	Loss of appetite, vomiting, low GI bleeding	10 N
3	69	M	DM, liver cirrhosis	No	Diarrhoea, fever and dyspnea	8 N
4	79	F	HT, CVD, HCV	Yes	Abdominal pain	8 N
5	63	M	No	No	Lower limb pain	> 70 N
6	62	M	Obesity, DM, HT, liver cirrhosis	No	Abdominal pain and vomiting	> 70 N
7	83	F	HT	Yes	Abdominal pain and dyspnoea	4 N
8	65	M	No	No	Abdominal pain	Not done
9	88	M	CVD	Yes	Abdominal pain	N
10	79	M	CVD	No	Cardiac failure	N
11	56	M	HCV, liver cirrhosis	No	Ascites	5 N
12	61	M	DM, HT	No	Dyspnoea	3 N
13	78	F	HT	Yes	Lower limb oedema	8 N
14	41	M	No	No	Fever and mental slowdown	6 N
15	64	F	HT	Yes	Fever and caught	2 N
16	89	F	Alzheimer disease	Yes	Caught and diarrhoea	N
17	59	M	Obesity, HT	No	Shortness of breathing	2 N
18	74	M	CVD, pharynx carcinoma	No	Loss of conscious	4 N
19	63	M	DM, HT	No	Fever, caught and dyspnoea	2 N
20	62	F	HCV, COPD	No	Melena and anaemia	Not done
21	72	M	Parkinson disease and dementia	No	Fever and dysuria	> 70 N

N: Normal value; ACT: Anticoagulant therapy; APT: Antiplatelets therapy; HT: Hypertension; CVD: Cardiovascular disorders; HCV: Hepatitis C virus; DM: Diabetes mellitus; COPD: Chronic obstructive pulmonary disease; GI: Gastrointestinal.

extended to the underlying wall. Necrosis was invariably accompanied by severe acute inflammation with diffuse micro-abscesses.

Findings of microvascular damage (endothelial shrinkage, leucocytes adhesion, vascular wall partial destruction) were reported in colonic (n. 3) and small bowel (n. 2) specimens. These features were studied in depth with CD34 staining for endothelial cells, and considered highly suggestive of SARS-CoV-2 infection related disease (Figure 3).

Outcomes

Surgery was necessary in four (19%) patients. Three had small bowel perforations repaired, and one small bowel resection. None of the surgeries was performed on the colon.

Median hospital stay was 9 d (interquartile range: 6-25). Mortality rate was 38% (95%CI: 0.18-0.62) as specified in Figure 4. No significant difference was found in univariate analysis when comparing patients stratified by outcome (Table 2).

Mortality among all the COVID-19 admitted patients was 23% (95%CI: 0.21-0.25). Mortality in COVID-19 patients with ischemic GI involvement had a relative risk for of 1.47 (95%CI: 0.007-0.031; $P = 0.31$), if compared to all COVID-19 hospitalized patients.

DISCUSSION

After the publication of several case reports or series of patients with COVID-19

Table 2 No significant difference was found in univariate analysis when comparing patients stratified by outcome

Variables	Recovered, *n* = 13	Dead, *n* = 9	*P* value
Age in yr[1]	65 (61-78)	71 (62-82)	0.63
Sex (F)[2]	6 (46%)	1 (11%)	0.17
ACT/APT[2]	5 (38%)	3 (33%)	0.99
D-dimer[1]	5 N (2 N-9 N)	5 N (3 N-54 N)	0.92
PT (INR)[1]	1 (0.9-1.3)	1.1 (1-1.5)	0.48
aPTT (INR)[1]	0.9 (0.8-1.1)	1 (0.9-1.3)	0.4
Platelets ($\times 10^9$/L)[1]	271 (226-325)	232 (68-278)	0.11

[1]Express in median (Interquartile range).
[2]Express in number of subjects (percentage).
F: Female; ACT: Anticoagulant therapy; APT: Antiplatelets therapy; PT: Prothrombin time; INR: International normalized ratio; aPPT: Activated partial thromboplastin time; N: Normal value.

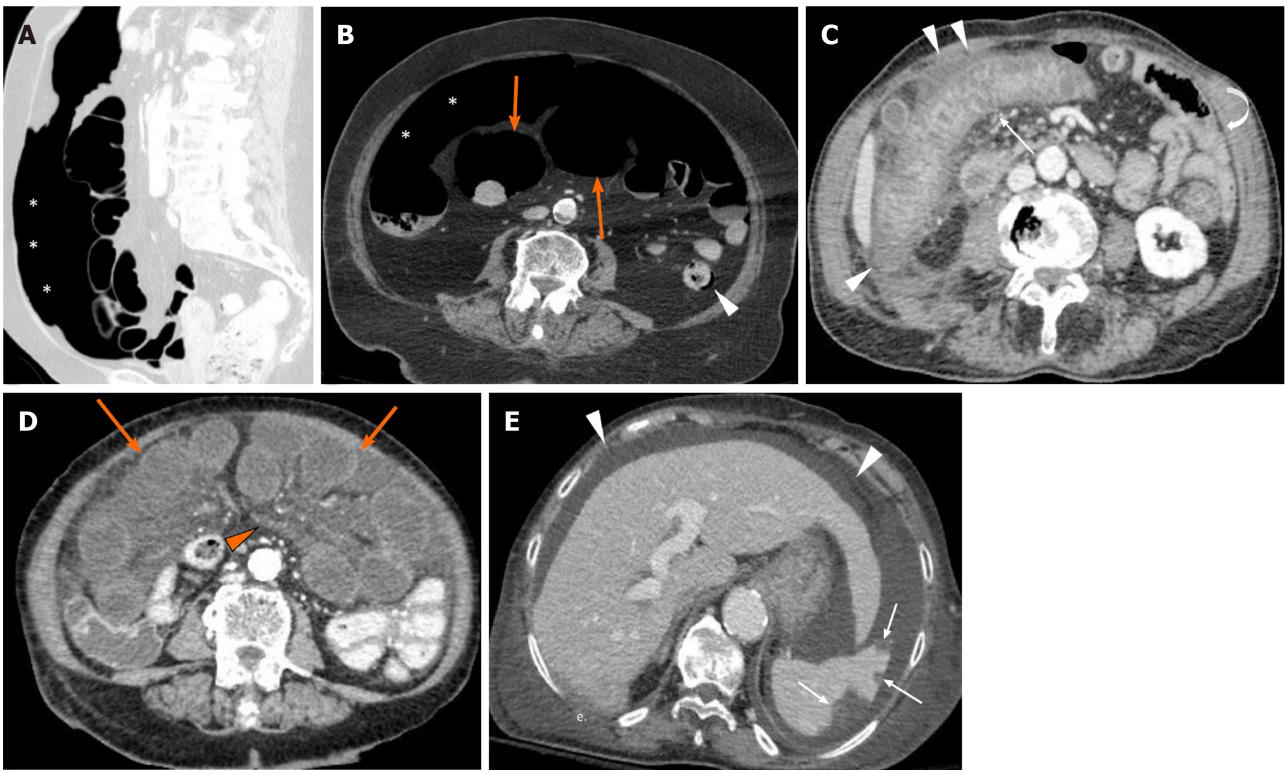


Figure 1 Computed tomography images from positive coronavirus disease 19 patients with abdominal signs and symptoms. A and B: The computed tomography (CT) scan revealed pneumoperitoneum (asterisks, A and B), dilated bowel loops with thin non-enhancing walls (orange arrows, B) and focal areas of pneumatosis in the descending colon (arrowhead, B), in keeping with intestinal ischemia. The main mesenteric vessels were patent (not shown); C: CT evidence of ischemic colitis involving the transverse and right portions of the colon: Markedly thickened and layered walls (thin arrow), compared to other normal appearing small and large bowel loops (curved arrow), with associated free fluid and oedematous stranding of the adjacent fat tissue (arrowheads); D and E: Axial CT scan shows multiple small bowel loops moderately dilated, with reduced thickness and poorly enhancing walls (orange arrows, D); concomitant diffuse oedema of the mesentery (orange arrowhead, D). In the same patient there were associated multiple peripheral splenic infarcts (withe arrows, E) with areas of mottled increased attenuation; the main splenic vessels were patent (not shown). Diffuse ascites (white arrowheads, E) is noted.

gastrointestinal ischemia[15-18], this multicentre retrospective study demonstrated that intestinal ischemic manifestations of SARS-CoV-2 were diagnosed in 0.7% of patients hospitalized with COVID-19 during the first pandemic wave. Ischemic signs must be suspected in all patients presenting to the ED with important abdominal symptoms and a positive SARS-CoV-2 swab, but they may also appear later as complications of SARS-CoV-2 pneumonia. Previous anticoagulant treatment does not seem to protect from the development of this complication. Contrast enhanced CT scan appears to be the best tool to promptly diagnose these manifestations and their

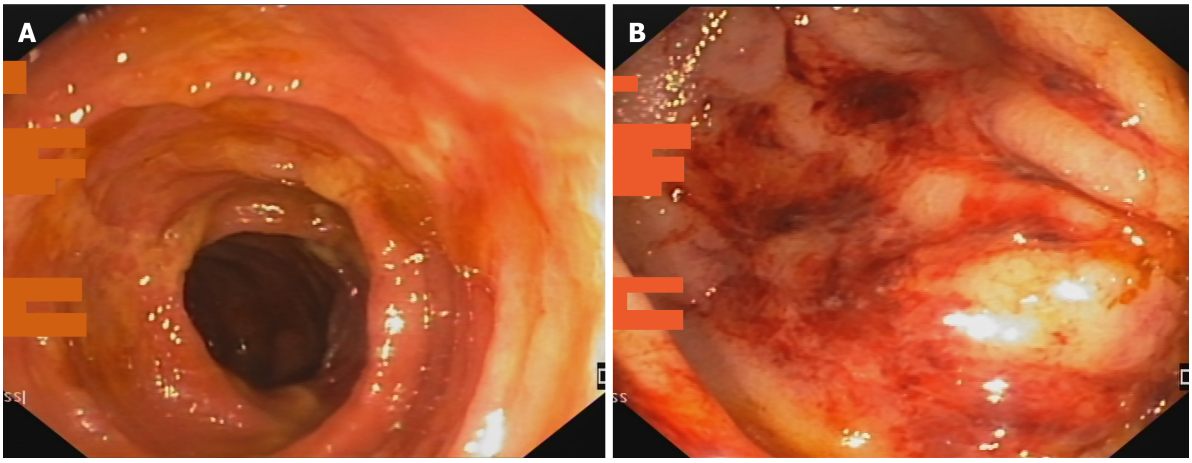


Figure 2 Endoscopic image of ischemic colitis. A: Oedematous mucosa with ischemic ulcerations; B: Presence of petechiae in an oedematous colic mucosa.

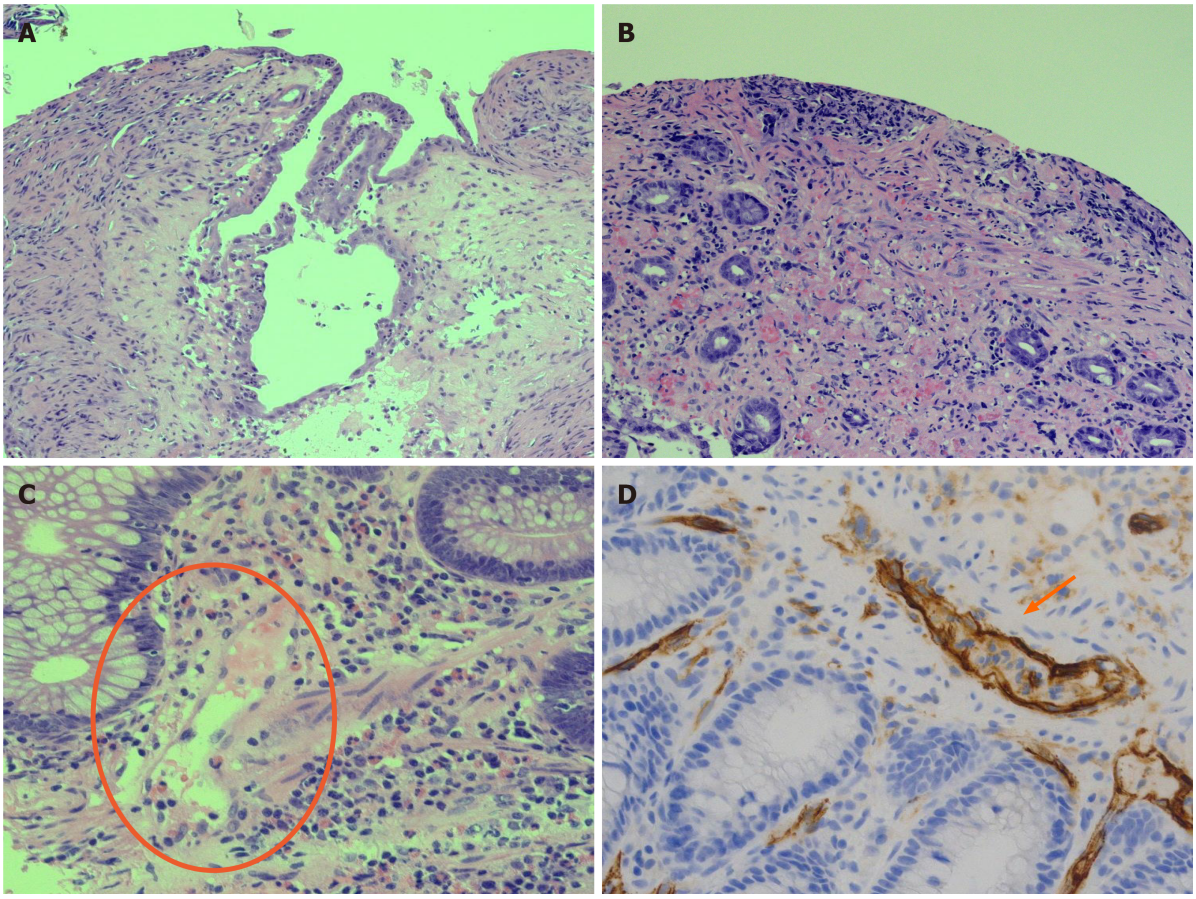


Figure 3 Histological features of ischemic damage of small and large bowel characterized by mucosal ulceration and fibrosis and vascular congestion of lamina propria associated with moderate mixed inflammatory infiltrate. A: Histological features of ischemic damage of small bowel; B: Histological features of ischemic damage of large bowel; C: Distinctive severe acute respiratory syndrome coronavirus 2 blood vessels inflammatory pattern with endothelial shrinkage and layering of granulocytes and lymphocytes on damaged vascular wall; D: Endothelial injury is emphasized by CD34 immunostaining of endothelial cells showing marked infiltration of inflammatory cells.

associated complications. Endoscopy is useful in case of suspected ischemic colitis. Atypical features on GI histology could guide the diagnosis even in patients with a SARS-CoV-2 negative nasopharyngeal swab. Nonetheless, a high index of suspicion must be kept, since mortality in these cases is as high as one in three patients.

Northern Italy and especially the Lombardy region were the most SARS-CoV-2 affected regions worldwide, with a seroprevalence after the first wave ranging from

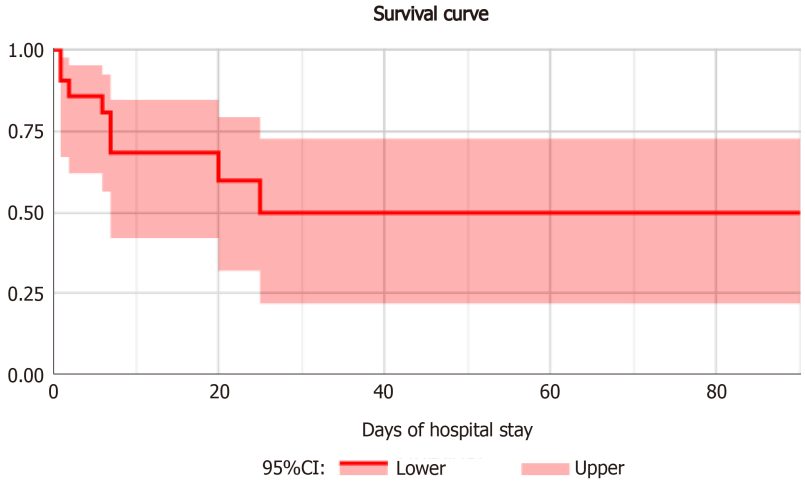


Figure 4 Survival curve of patients with intestinal ischemia.

24% to 38.5%[19-21], and an hospitalisation rate < 1%; thus, intestinal ischemic manifestations seem to be a very rare complication of SARS-Cov-2 infection.

Although the median hospital stay of the studied patients is similar to that of other larger cohorts of COVID-19 patients[22,23], the mortality is confirmed to be higher[22, 23]. Furthermore, if we consider the latest results from Imperial College on Infection Fatality Rate (2.3% in SARS-CoV-2 infected patients in Italy)[24], intestinal ischemic manifestations impact with an over 15 times higher lethality.

As already highlighted by Bhayana and colleagues, the radiologists play a central role in the diagnosis of these complications, with intestinal wall abnormalities described in nearly a third of abdominal CT scans performed in COVID-19 patients admitted to the intensive care unit (ICU)[25]. The main relevant finding in our cohort of patients is that in almost all patients with CT signs of bowel ischemia or colitis/enteritis there was no evidence of acute venous or arterial vessels thrombosis. Only one of six patients with bowel ischemia showed concomitant acute IVC and SMV thrombosis. This is in line with the report from Bhayana[25]. Interestingly, in 4/20 patients CT scan showed splenic (3) or renal (1) parenchymal infarcts, but only in one case we identified splenic artery thrombosis. The rate of complications related to bowel involvement was relatively low, accounting for CT scan evidence of perforation in one case and loops over distension in 6/20 patients. Bowel over distension, with no evidence of obstructing aetiologies, was therefore most likely functional.

Endoscopy may help confirming the diagnosis of patients with ischemic colitis, as outlined by the early published reports[26,27], although caution is mandatory when performing endoscopy in unstable patients admitted to ICU[28]. This is in agreement with a multicentre study on endoscopic findings of SARS-CoV-2 positive patients during the first wave in northern Italy, which showed histologically confirmed colon ischemia as one of the most prevalent findings[29].

In our series the histological analysis of colon biopsies together with small bowel resected segments showed very distinctive aspects of endothelium inflammation and endotheliitis, which were firstly described in the pulmonary, renal and gastrointestinal tract of SARS-CoV-2 infected patients[30]. These findings may give an explanation to the previously mentioned radiological evidence of ischemic damage without main venous or arterial thrombosis. Furthermore, in our cohort they may explain the absence of topographical segmental and patchy distribution of endoscopic ischemic lesions, which are usually expression of occluded vasculature[13].

The strong tropism of SARS-CoV-2 for the gastrointestinal tract is probably mediated by the abundance of Angiotensin Converting Enzyme 2 receptors in the intestinal mucosa[31]. A possible explanation for the virus induced endothelial damage in the gastrointestinal tract could lie in the lectin pathway, which is supposed to be responsible for SARS-CoV-2 mediated thrombotic microangiopathy in lung tissues[32]. The central role of Lectin and mannan-binding lectin-associated serine protease-2 in the gastrointestinal ischemic reperfusion damage has already been described in murine models in the pre-COVID-19 era[33,34]. These findings, if confirmed, could open new interesting therapeutic fields of research on SARS-CoV-2 ischemic manifestations.

CONCLUSION

Despite several limitations, mainly due the retrospective design on an infrequent and previously unknown manifestation of SARS-CoV-2, this multicentre study was able to confirm early isolated observations of gastrointestinal ischemia. Furthermore, new findings on epidemiology, clinical presentation, distinctive diagnostics features and outcomes were illustrated and discussed. This preliminary results in a small series of patients might raise the awareness on an infrequent but potentially lethal manifestation of COVID-19, aiding clinicians in proper management and prognostic stratification of complicated cases.

ARTICLE HIGHLIGHTS

Research background

Clinical manifestations of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus are heterogenous and can affect different organs, including the gastrointestinal tract. Angiotensin converting enzyme 2 receptor, which mediates SARS-CoV-2 infection, is abundantly present in the intestinal mucosa.

Research motivation

Since the cytokine storm mediated by SARS-CoV-2 in coronavirus disease 19 (COVID-19) seems to determine a vascular damage which could explain hypercoagulability and pulmonary embolism, intestinal ischemic events in SARS-CoV-2 positive patients may be linked to the same pathogenic mechanism.

Research objectives

The aim of the present study is to collect and analyse the intestinal ischemic events in SARS-CoV-2 positive patients in order to calculate the incidence and determine the prognosis of affected subjects.

Research methods

The study was designed as a retrospective observational multicentre collection involving three among the largest COVID hospitals in Lombardy.

Research results

Intestinal ischemia is a rare but fatal manifestation of SARS-CoV-2 infection. The condition should be suspected in case of severe abdominal pain. In order to define localization and extent of intestinal ischemia, abdominal computed tomography scan and possibly endoscopy should be carried out. Intestinal biopsies main finding is endotheliitis.

Research conclusions

Severe endotheliitis in the intestinal mucosa could mediate intestinal ischemic manifestations in the gastrointestinal tract. Such manifestations are rare but frequently fatal, thus they should be ruled out in SARS-CoV-2 positive patients with gastrointestinal symptoms.

Research perspectives

One of the possible mechanisms of ischemic intestinal manifestations of SARS-CoV-2 could be mediated by mannan-binding lectin-associated serine protease-2 which is also a possible target for future therapy of COVID-19.

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